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THE SIGNIFICANCE OF THE ASYMMETRY OF THE OVARIES OF THE FOWL[1]

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FIVE HELIOTYPE PLATES (EIGHTEEN FIGURES)

AUTHOR'S ABSTRACT

A chronological investigation of the histological condition of the right ovary of the domestic hen from embryos of nine days' incubation to adults of eighteen months was made. A rudiment of the right ovary is found at all times. Its composition is variable. Sixty-one per cent of the rudimentary ovaries contained medullary tissue only. Thirty-nine per cent had rudiments of cortex, in addition. The occurrence of cortical rudiments in embryonic stages is the probable basis of ovarian follicles found in the rudimentary right ovaries of adults. Primordial germ cells persist in the medullary tissue until three weeks after hatching. They subsequently appear to atrophy. The medullary cords persist through the entire period either as distended tubules or as solid cords of modified epithelial cells. Remnants of the right mesonephros persist as tubules and connect with the gonad by rete tubules. The mesonephric duct maintains a patent lumen.

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INTRODUCTION

The domestic fowl normally possesses but one functional ovary which is located on the left side of the body. A rudi-

ment has been described as occurring on the right side by Chappellier ('14), Stieve ('24), and others.

In recent years, experiments in which the ovary has been removed have called attention to the problem of the asymmetry of the gonads of the fowl. In this laboratory Domm ('27) has ovariotomized some 121 light brown Leghorn females. The results of the operation have been as follows: Accompanying the removal of the left ovary, the head furnishings reduce and the plumage assumes the male-like appearance typical for capons of the breed, the spurs grow, and a compensatory growth begins to develop on the site of the right rudimentary ovary. This growth usually resembles a testis, but has a broader attachment than a testis does normally. When this testis-type compensatory growth reaches a condition of active secretion, the plumage reverts to the female type. Other secondary changes also occur, namely, the head furnishings enlarge to male size and form and the sex behavior becomes more like that of the male. Fifty of these compensatory growths were examined histologically, and their appearance resembled that of an immature testis in many respects. There were tubules lined with columnar epithelium, but in no cases were there any traces of spermatogenetic tissue. In other rare cases an ovary developed on the right side instead of the testis-like compensatory growth, and in still other cases an ovotestis was found. Associated with these exceptional growths were corresponding exceptional responses on the part of the operated bird which have not yet been fully described.

Previous investigators of the problem, such as Goodale ('16), who first performed the experiment, Zawadowsky ('22, '26), Benoit ('23 a), Pezard, Sand and Caridroit ('25), and Finlay ('25), have described the occurrence of an hypertrophied body on the right side following ovariotomy. In the two cases described by Benoit and in one case of Zawadowsky spermatogenesis was reported. In regard to his experimental results, Domm ('27) says:

The most significant feature of our histological studies when reviewed in the light of Benoit's, Zawadowsky's, and Finlay's, is that none of the compensatory growths studied by us showed spermatogenesis nor was there anything comparable to normal spermatogenetic tissue found. This is really significant when one considers the fact that the only two cases studied by Benoit ('23 a) both showed normal spermatogenesis in the right compensatory gonad, and Zawadowsky ('26) describes one case showing normal spermatogenesis in a relatively small number of ovariotomized birds, while in none of the right compensatory gonads that we have studied (fifty cases) nor in those of Goodale ('16) and Greenwood ('25) was there any indication of the onset of spermatogenesis.

The compensatory growth, whether of testis type, ovotestis, or ovary, always rises on the same site, namely, that homologous to the left ovary. Its variable character requires explanation. The purpose of the present investigation is to ascertain what data of the normal embryonic history are applicable to this problem.

In examining twenty adult birds to determine the nature of the right rudiment, I discovered two cases of well-developed right ovaries containing distinctly visible follicles. Domm ('27 and unpublished data),[2] working in this laboratory, reports that, among several hundred female brown Leghorns examined, twenty-five cases showed development on the right side of a somewhat normal ovary containing follicles. The completeness of development of ovary which may occur on the right side in the hen is shown in Domm's bird no. 726, in which on the right side was "a mass of ovary showing two large yellow follicles about ready for ovulation, with many smaller ones of varying sizes. Two ruptured follicles, in the form of shrunken sacs were found." In six other cases the right body was an ovotestis, in part resembling a testis, yet also containing ovarian follicles. In examining seventy-one operated birds, Mr. F. W. Appel (unpublished data),[2] working in this laboratory, has observed three cases of well-developed right ovaries and eight cases in which there was an ovotestis on the right side.

Comparatively rare occurrence of ova in the right gonad of birds and variable development of the gonad under normal conditions have been observed by several authors. His ('68) described right ovaries possessing ova in the domestic fowl and in pigeons. He refers to earlier citations of the occurrence of right ovaries in birds. Chappellier ('14) described a domestic duck (Anas boschas var.) of unusual interest, in which both the right and left ovaries and the right and left oviducts were well developed and functional. Calyces indicating extrusion of eggs were present in both ovaries. Chappellier ('14) made a very comprehensive study of the literature regarding occurrence of the right ovary and right oviduct in birds. He showed that the phenomenon is widespread, existing in birds of ten orders and in forty-four species of twenty-nine different genera. Fourteen cases in which the right ovary is larger than the left were reported. Two cases were cited in which no left ovary was present. Riddle ('17, '18, '25) has observed many instances in pigeons in which there were right ovaries with ova. Stieve ('24) describes a considerable number of new cases among birds of prey in which there were right ovaries with follicles. Exceptional development of the right ovary was found in two chicken hawks (Falco palumbaris Kl.). In one of these cases both ovaries were of similar size. The right ovary as well as the left was functional, for, in addition to follicles, there was a calyx which indicated that an egg had been extruded from the right ovary. Only a small rudiment of the right oviduct was visible near the cloaca. Stieve concluded that the egg from the right ovary was laid from the left oviduct.

In looking through the literature for a possible explanation of this series of phenomena on the basis of the embryological history of the right ovary, one is surprised to find that its complete history has not been followed. Stieve refers to the fact that both ovaries are found in the embryonic stages of many birds. In young jackdaws at the time of first flight he found in 5 per cent of the cases small right ovaries with a few little follicles, but in old birds the right ovary disap-

peared almost always completely. The general statement has been that the right ovary undergoes total degeneration shortly after hatching. Even Firket ('14, '20), who made a very accurate survey of the early embryonic history of the gonads of the fowl, found in his material no indication of the possible persistence of the rudiment of the right ovary. His conclusion was:

> Toute la masse subit la dégénérescence graisseuse analogue à celle déjà décrite pour l'ovaire gauche; cette dégénérescence aboutit à la disparition complète de tous les éléments constitutifs de l'ovaire droit; seul l'épithélium superficiel est conservé, mais il ne se distingue plus du restant de l'épithélium péritonéal ('20, p. 400).

However, to explain the experimental results and anomalies of development cited, it would seem necessary to assume the continued existence of the right ovary as a persistent rudiment of variable composition. In view of the need for knowledge of the normal embryology and histology of the right ovary, with special reference to the variable experimental results, this investigation was undertaken.

RÉSUMÉ OF THE RESULTS OF THE INVESTIGATION

The results of the investigation go far to explain the variations noted above. A brief statement of the findings is as follows:

The right ovary is observed to retrogress slightly after hatching, but to persist as a rudiment which grows further in adult life. The rudiment is of variable composition. In most cases there is medullary tissue only, while in other rare cases cortical cords and ova are also found. The realization of the expectation of finding cortical tissue in the embryological stages of the right ovary was previously anticipated by Willier ('27). In the present investigation, however, varying amounts of cortical tissue in the right ovary are described in a series of embryos and chicks from nine days' incubation to adult hens. In an effort to determine the embryological basis for the histological constitution of the right compensatory growth, the occurrence and condition of

primordial germ cells in the right ovary was observed chronologically. The primordial germ cells are relatively abundant in early stages, but practically disappear from the rudiment at about three weeks after hatching.

Material and methods of study

The early embryonic history of the gonads of the fowl has been described adequately by Firket ('14, '20) and Swift ('15, '16). The point of departure for my investigation is the embryo of nine days' incubation. Beginning with this stage, the gonads have been examined in female birds at frequent intervals to sexual maturity at six months and at the age of a year and a half. A number of specimens of each age have been examined both anatomically and histologically, in order to form a somewhat quantitative estimate of the variability of the normal development.

Measurements of the size of the embryonic gonads were taken by a millimeter rule. The lengths of the ovaries of chicks, pullets, and hens were estimated from serial sections. Thickness of tissues and sizes of cells were calculated from sections by readings of an ocular micrometer.

All of the material used has been of the brown Leghorn race. The embryos and chicks were fixed in Bouin's picroformol fixing fluid. The sections were cut 6 and 8 μ thick. The staining method used was Heidenhain's iron hematoxylin. It was not the aim of the investigation to make a refined cytological study of either primordial germ cells or other classes of cells.

Early embryonic history of the gonads

As an introduction to the description of later stages the present state of knowledge in regard to the early embryonic history of the gonads is first summarized. Since an essential part of the investigation deals with the occurrence and ultimate fate of the primordial germ cells from which the definitive sex cells develop, a statement of the criteria used for their identification is included also.

The gonads of the fowl develop in conjunction with the mesonephroi. They are visible in the opened body cavity of embryos of three and three-fourths days' incubation (ninety hours) as elongated whitish ridges, about 1.5 mm. long, which lie on the medial surfaces of the mesonephroi. The ridges begin slightly posterior to the anterior ends of the mesonephroi and extend over their anterior halves. The gonads in both sexes are asymmetrical even at this early stage, the left being more massive than the right (Swift, '15).

At this time the flat cells of coelomic epithelium have already differentiated on the surface of the gonads as a single layer of elongated cylindrical cells possessing oval or round dark-staining nuclei. The differentiated tissue with included primordial germ cells is called the germinal epithelium (Bornhaupt, Waldeyer, '70). During the following two days it becomes two or three cells thick and is distinctly limited by a basement membrane.

The mass of the gonad underlying the germinal epithelium consists of a stroma of closely packed mesenchyme. In this stroma appear sixteen solid cords of cells known as the cords of urogenital union or rete cords (Firket, '14). They extend obliquely from renal corpuscles of the mesonephros to positions under the germinal epithelium. The cells of the cords are directly continuous with those of Bowman's capsules from which they originated (Hoffman, '92). The fate of these cords is to form the rete, which is especially conspicuous in the testis where it ultimately forms the vasa efferentia. Throughout the gonad, but especially in the germinal epithelium, is another tissue element, the primordial germ cells. They have been present from the origin of the gonads and even earlier. Their history has been worked out by Swift ('14). In brief, according to this author, it is as follows:

The primordial germ cells originate from the germ-wall entoderm anterior and anterolateral to the embryo during the primitive-streak stage and up to the 3-somite stage. From this place of origin, by means of amoeboid movement, they enter the mesoderm of the vascular area and are carried in

the blood vessels to all parts of the embryo. At the stage of 20–22 somites the primordial germ cells leave the vascular system, and a great number of them lodge in the mesoderm of the splanchnopleure near the coelomic angle. At three days incubation, they are distributed in the radix mesenterii, the coelomic epithelium, and in the mesenchyme beneath on both sides of the coelomic angle.

When the germinal epithelium is differentiated, numerous primordial germ cells are included in it along with the cells of peritoneal origin. Firket ('14) first called attention to a striking inequality in their distribution. The left gonad in both sexes receives two to five times more than the right does. At five and one-half days, due to local increase of the epithelial cells, buds or projections begin to grow from the germinal epithelium into the underlying stroma of the gonad. This activity, which reaches a maximum on the sixth day and ends at six and a half days, results in the proliferation of a series of epithelial cords, which are known as the cords of the first proliferation. In the male these are the true sexual cords or seminiferous cords, while in the female they become the medullary cords. These cords of the first proliferation become separated from the germinal epithelium and continue to increase in size, the process occurring more rapidly in the female gonads than in the male gonads. The bulk of gonadal tissue under the germinal epithelium is composed of these cords. The two kinds of cells of the cords at this time may be distinguished as primordial germ cells and epithelial cells of peritoneal origin.

This marks the end of the indifferent period, during which time the sex of the embryos has been indistinguishable morphologically. After this time, six and a half days (156 hours), a characteristic development is followed, respectively, by the left ovary, the right ovary, and by the testes.

A brief synopsis of the known history of development of the gonads after sex differentiation follows:

Testes. In the gonads of males the cells of the germinal epithelium very quickly become cubical in shape and are

reduced to a single layer. Hardly a single primordial germ cell remains in the epithelium, nearly all being included in the sexual cords which form the definitive seminiferous tubules. The sexual cords remain thin and attached to the germinal epithelium until after nine days, then they are entirely constricted off. Between the cords and the epithelium the connective-tissue stroma develops into a broad band, the definitive tunica albuginea. Elsewhere the stroma forms a kind of mesenchymal syncytium between the cords. The sexual cords continue to grow by mitosis of the epithelial cells. They increase in length, become convoluted, and later increase in diameter and anastomose so that they form a reticulum with large interstices. After thirteen days the primordial germ cells begin to increase in numbers mitotically, and concurrently the mitochondria become grouped around the attraction sphere; this marks their transition into spermatogonia. After twenty days' incubation, the spermatogonia which were previously scattered at random throughout the cord become arranged against the basement membrane, with one to three epithelial supporting cells between them. Lumina develop in the centers of the cords which now begin to resemble the definitive tubules.

Left ovary. After the formation of the medullary cords ceases, the germinal epithelium of the left ovary, which has continued to increase in thickness, has several more layers than that of the male gonads or the right ovary. The number of primordial germ cells is greater in the germinal epithelium of the left ovary than that of the right ovary or the testes. This is accounted for by the fact that the increased thickness of the germinal epithelium removes many of the primordial germ cells from inclusion in the proliferating medullary cords (Swift, '15, p. 460). This tissue is called cortex and is sharply distinguished from the underlying medulla which consists of medullary cords. A layer of connective tissue, the primary tunica albuginea, separates the two regions.

At eight days, there begins a period of rapid multiplication of the primordial germ cells in the cortex, which indicates the transition into oogonia. The mitochondria have assumed at this time a characteristic arrangement about the centrosphere. The oogonia continue to divide through the ninth, tenth, and eleventh days. Their activity results in the formation of the cords of the second proliferation, or the cortical cords from which the definitive ova and their follicular cells develop. These cords separate from the germinal epithelium which becomes reduced to a single layer of cubical to columnar cells. Connective tissue then forms the definitive tunica albuginea.

Right ovary. The normal history of the right ovary resembles that of a testis in the absence of ingrowth of cords of the second proliferation. After the cords of the first proliferation are formed, the germinal epithelium remains thin, as a single layer of cuboidal cells, among which are a very few primordial germ cells. There is ordinarily no secondary proliferation of cortical cords such as occurs in the left ovary. The medullary cords continue to grow as they do in the left ovary, but more slowly. Throughout its development the right ovary is much smaller than the left. The further history of the right ovary will be considered in detail subsequently. As previously noted, Firket maintains that the right ovary degenerates completely.

Primordial germ cells. An important part of this investigation has been concerned with the prevalence and condition of cells known as primordial germ cells in the ovary (Waldeyer, '70). For the identification of these cells, criteria established by Firket ('14) and Swift ('14) have been used. In brief, the criteria are as follows:

A single description will cover the condition of the primordial germ cells from the time of their origin to their differentiation into oogonia. Change in their structure is limited to one or two features. The primordial germ cells are much larger in size than neighboring cells. Their average diameter is 16 µ, although they may vary from 14 µ to 22 µ. Their shape, which is round or oval, is characteristic.

The nucleus is large, 8 μ to 12 μ in diameter. It is spherical and vesicular, with a definite nuclear membrane. The nucleus is clearer than those of other neighboring tissue cells, although the amount of chromatin is about the same. The greater size of the nucleus causes this appearance. The chromatin in the nucleus has a characteristic arrangement in two masses of granules, sometimes connected by threads of granules or separated by a clear interval. In the earliest stages the chromatin is in the form of a reticulum. In the later stages approaching synapsis the arrangement becomes that of the various phases of meiosis.

The nucleus is eccentrically placed in the primordial germ cells. At the pole where there is a wide expanse of cytoplasm there is located the attraction sphere. The attraction sphere, which is a striking diagnostic character especially in the primordial germ cells, appears as a large condensed or flattened sphere of cytoplasm resting on, or slightly separated from, the nuclear membrane. It is from 3 μ to 6 μ thick and often is markedly discrete.

Yolk remains in the younger primordial germ cells for some time in the form of spherules, but is mostly digested by the ninth day of incubation. This retention of yolk is probably related to the absence up to this time of mitoses in these cells (Swift). Evidence of mitoses in primordial germ cells is first conspicuous about the eighth day of incubation.

Mitochondria, usually in the form of short rods, are present in the primordial germ cells. Up to the seventh day of incubation, they are evenly distributed throughout the cytoplasm. Following this, they are characteristically grouped in the form of a crescent or cap in the region of the centrosphere.

Another limiting character of the primordial germ cells is their extragenital origin and subsequent migration into the gonads. This fact was demonstrated by Swift ('14) and has recently been confirmed by Richards, Hulpieu and Goldsmith ('26). A description of the origin and occurrence of the primordial germ cells has been given above.

My use of the term primordial germ cell is restricted to cells of this description. For the purpose of this investigation, I have not attempted the finest methods of cytological technique for the differentiation of these elements. The diagnosis, however, is in every case unmistakable, except where uncertainty is expressed. The material does not permit a definite stand on the question of the possible peritoneal origin of definitive sex cells.

Details of the investigation

The details of the investigation have been divided into periods as follows:

Embryo: Nine to fourteen days' incubation.

Embryo: Fourteen days' incubation to hatching.

Chick: First three weeks after hatching.

Chick: Three weeks to six months.

Adult hens: One year and six months old.

The observations under each period, where the material permits, are organized under the following headings:

Size and shape of the gonads.

Anatomical relations of the gonads.

General histology of the gonads.

Cortex of the left ovary.

Cortex of the right ovary.

Medulla of the left ovary.

Medulla of the right ovary.

Primordial germ cells in the medulla of the left ovary.

Primordial germ cells in the medulla of the right ovary.

Interstitial elements of the left ovary.

Interstitial elements of the right ovary.

Mesonephros and mesonephric duct.

Embryo: nine to fourteen days' incubation. In the study of this stage forty-three embryos have been examined. Serial histological preparations have been made of the gonads of twenty-nine. Of these, eleven were of the right ovary only. Fourteen have been measured only.

Size and shape of the gonads. When the viscera are removed from the body cavity, the gonads are seen to be conspicuously white bodies lying on the ventromedial surface of the pink mesonephroi. The disparity in size between the right and left ovary is well marked at nine days' incubation. In ten embryos examined, the left ovary was larger in every case. Similar asymmetry was observed in all other embryos examined in the entire investigation. The length of the right ovary at nine days' incubation, averaged from measurement of five specimens, varied from one extreme of 1.4 mm. to 2.0 mm. at the other, with the average length 1.5 mm. The left ovary of five embryos measured, varied from 2.0 mm. to 3.0 mm. in length. In seven embryos of ten days' incubation measured, the right ovary averaged about 2.0 mm. The left ovary of eight embryos measured, varied from 2.5 mm. to 4.0 mm. In one embryo of eleven days' incubation measured, the right ovary was 1.5 mm. long. The left ovary of two specimens measured 3.0 mm. and 4.0 mm., respectively, in length. The shape of the ovaries is illustrated by specimen E 124 of ten days' incubation (fig. 1). Externally, this specimen shows slight lobulations of its surface. Occasionally right ovaries show more pronounced lobulations which are correlated with development of cortical tissue beneath the prominences.

Anatomical relations of the gonads. The three-lobed metanephros lies against the dorsal wall on each side of the spinal cord in the embryo of the fowl. The dorsal aorta lies between them. The two mesonephroi lying ventral to the metanephroi conceal most of the first lobes. The adrenal gland is triangular in shape and fits into a medial concavity at the extreme anterior end of each mesonephros. The two iliac veins pass obliquely over the ventromedial surfaces of the mesonephroi and by a wide connection join together to form the postcaval vein about midway of the lengths of the mesonephroi. The postcaval vein then inclines to the right, passes over the ventral surface of the right adrenal gland, and enters the liver.

The left ovary conceals the left adrenal gland, beneath which it lies and extends posteriorly over the surface of the left mesonephros in a slightly medial direction. It lies partly on the surface of the postcaval vein. The posterior third of the ovary is folded dorsally, causing the gonad to assume a more medial position posterior to the conjunction of the iliac veins.

The right ovary begins anteriorly along the right lateral side of and partly extending onto, the postcaval vein as a narrow indistinct body which tapers posteriorly. It attains clearer definition and maximum width at the level of the conjunction of the iliac veins. The position of the right ovary is even more oblique than that of the left. It begins at a considerable distance behind the anterior extremity of the left ovary, toward which it extends at a marked angle until reaching the medial side of the right mesonephros, along which it lies nearly to the level of the posterior end of the left ovary. The flexure of the right ovary is paralleled by an equally characteristic bend in the right testis (figs. 2, 3, and 4).

Histology of the gonads. The histological description of the ovaries of this stage is based on a detailed examination of twenty-eight embryos. Eight of nine days' incubation, two being preparations of the right ovary only; ten of ten days' incubation, three right ovary only; nine of eleven days' incubation, six being right ovary only; and one of twelve days' incubation.

In the left ovary at the age of nine days' incubation there is already indication of a cortex, which covers the entire free surface, and a medulla. The cortex consists chiefly of a germinal epithelium with groups of primordial germ cells. The medulla shows two distinct layers, a superficial layer beneath the cortex which merges into the second which has the form of a reticulum with large interstices. The cortex is separated from the medulla by a thin layer of connective tissue which forms the primary tunica albuginea. The right ovary differs decidedly in appearance from the left ovary.

There is, in general, no evidence of origin of a cortex and the medulla shows less of the compact and more of the reticular constitution.

Cortex of the left ovary. The germinal epithelium of the left ovary is columnar and varies in thickness from one to four cells. It is sharply defined by a basement membrane and is separated from the medulla by connective-tissue stroma of the primary tunica albuginea. In between the epithelial cells are numerous primordial germ cells. As many as ten or twelve can be seen in a single low-power field. Some of these cells show mitotic figures. The epithelium is not of uniform thickness. In places lobulations extend from its deep surface into the medulla. These indicate the genesis of cortical cords, or the cords of the second proliferation (fig. 7).

Cortex of the right ovary. The occurrence and arrangement of cortical material in the right ovary is highly variable. The surface of the ovary was in every case covered for almost its entire area by a cubical epithelium. In places the cells were much flattened or squamous. In practically every embryo it was not uncommon for primordial germ cells to lie among the small flattened cells of the epithelium. Usually they were isolated. At times two or three were adjacent to each other (fig. 8). Firket cites and illustrates this phenomenon as being very exceptional ('20, fig. 9, pl. VII). Swift ('15) states that in the male gonads primordial germ cells are hardly ever left in the germinal epithelium after the cords of the first proliferation are constricted off. He cites Hoffman ('92) to the same effect. Such occurrence of primordial germ cells does not constitute cortex, although it is indicative of a possible tendency in that direction. The epithelial cells near such primordial germ cells are often more columnar than elsewhere.

In a few of the embryos examined the right ovary showed sporadic patches of unmistakable ovarian cortex. In such patches extending through 50 μ to 200 μ the cells of the germinal epithelium were columnar and several layers thick.

Among the epithelial cells primordial germ cells were so numerous that the appearance was identical with that of the normal cortex of the left ovary (fig. 9). At the deep surface of such groups of primordial germ cells there often was a distinct basement membrane. There were cases of lesser degree of cortical development also, in which the germinal epithelium was single-layered and the cells less cylindrical. Some embryos showed a still more advanced condition. The germinal epithelium was columnar, cubical, or squamous, but constricted off from it and lying next to the medulla were occasional groups or cords of from four to twenty and more primordial germ cells. They were surrounded by a basement membrane and a layer of connective-tissue stroma. Included in these cords with the oogonia were a few small epithelial cells. Cords of this type probably represent a stage later in development and leading to the type of large cortical cords found in the embryo at the age of fourteen and fifteen days. While it might be disputed whether these cords of cells originate from the second proliferation or not, it seems very certain that they are cortical cords which are detached from the germinal epithelium. The arrangement of the mitochondria is in the crescentic cap characteristic of oogonia. Accordingly, I have catalogued specimens of this nature as possessing cortical rudiments. In none of the cases was primary tunica albuginea developed to such an extent as in the left ovary. It was often difficult to find any trace of albuginea.

The data with regard to the presence of cortex in the right ovary of embryos of this stage may be summarized in statistical terms as follows:

Of the eight embryos of nine days' incubation studied histologically, two showed unmistakable evidence of cortex, three showed a possible tendency toward such formation and three showed no trace of cortex. Of the ten embryos of ten days' incubation, three showed cortical rudiments, one showed a cortical cord constricted off from the germinal epithelium, and six showed no indication of cortical rudiments. Of the nine embryos of eleven days' incubation three showed patches

of cortex, one showed a well-defined cortex and also a large cord in the medulla. The total percentage showing cortical rudiments out of the twenty-eight examined was approximately 33 per cent. This appearance of scattered rudiments of cortex in the right ovary in such a large proportion of the embryos examined was unexpected.

Medulla of the right ovary. The medulla of the right ovary has the same cellular elements which have been described as composing the medulla of the left ovary. The structural arrangement varies from the uniform character of the left medulla and also varies in different specimens. In all cases the texture is much looser than that of the left medulla. There is no region of solid cords of epithelial cells, but distended cords lie against the germinal epithelium itself (fig. 11). Solid areas are less numerous and isolated. This condition bears no relation to any particular region or level of the gonads and varies in different specimens. In one case, specimen E 305, the medulla is much more solid than in the others and resembles in texture the left ovary. Where cortical rudiments are well developed, the underlying medullary tissue is correspondingly more compact than elsewhere. In the right ovary both the solid and the distended medullary cords are more clearly differentiated than in the left ovary. As a rule, the epithelial cells of distended cords are very squamous. At the region of attachment of the right ovary to the mesonephros the distended medullary cords are smaller than the correspondingly placed cords in the left ovary. Mitotic activity is often found in the epithelial cells of the medullary cords. Isolated epithelial cells are common and may be found in the process of division. The isolated cells are much larger than those in the cords.

Primordial germ cells in the medulla of the left ovary. Primordial germ cells are found as constituents of the medullary cords, but usually not in the compact part of the medulla. Where the cords are partially distended and clearly defined, one or two may be found in the epithelial wall of the tubule. In other cases the epithelial cells closely surround a single

primordial cell, or a small group of two or three are included in a cord of epithelial cells. Very often a single primordial germ cell or a group of two or three is seen loose in the lumen of a larger tubule whose wall has become flattened or squamous epithelium. Primordial germ cells are also found isolated or in groups of two or three among the intercordal or intertubular cells. Occasionally mitotic figures are found in primordial cells in the medulla.

Primordial germ cells in the medulla of the right ovary. The number of primordial germ cells varies. In some ovaries there are only a few in a section, while in others they are conspicuously abundant, as many as ten or twelve in a single low-power field, or twenty to twenty-five in a section. The primordial germ cells appear to be relatively more numerous than in the medulla of the left ovary. In the latter they are restricted to the trabecular part of the medulla. The primordial germ cells occur in the same manner as in the left medulla, namely, loose in the lumina of distended cords, in the walls of distended cords, in solid cords with other epithelial cells, and isolated.

Interstitial elements of the left medulla. In addition to the cellular components mentioned, there are in the medulla connective-tissue cells of the stroma, blood sinusoids often filled with erythrocytes, and numerous small round dark-staining 'primitive wandering cells,' and less numerous oval eosinophilic granular leucocytes. The connective-tissue cells of the stroma give no indication of a differentiation as secretory cells.

Interstitial elements of the right ovary. Intercordal or intertubular tissue in the right medulla is similar to that of the left medulla. It includes connective-tissue cells of the stroma, erythrocytes in blood sinusoids, numerous primitive wandering cells and granular leucocytes.

Embryos of fourteen days' incubation to hatching. Size and shape of gonads (compare figs. 3 and 4). Thirty-five embryos at this period of development were examined. Measurements were taken of thirty-one and histological

preparations were made of nine. Both the left and right ovaries have increased in size, but the left has grown relatively much more than the right ovary. The left ovary varies in length from 3.5 mm. to 7.0 mm. The majority measure about 5.5 mm. in length and 1.5 mm. in width. The right ovary varies in length from 1.2 mm. to 3.5 mm. Fourteen measure 2.0 mm. in length. The width varies from 0.5 mm. to 0.9 mm.

Anatomical relations of the gonads. The positions of the gonads with respect to surrounding viscera are practically the same as at nine days' incubation. The right ovary lies obliquely along the lateral edge of the postcaval vein, reaches its greatest width at the level of the conjunction of the iliac veins, and then lies along the median surface of the right mesonephros. The right ovary varies in external appearance in different embryos. Some are poorly differentiated from the adjacent mesonephric tissue, while others are well defined. Two specimens of fifteen days' incubation, E 140 (fig. 3) and E 464 and one of sixteen days' incubation are remarkable for a distinctly lobed appearance and a more opaque and compact texture. As will be noted later, histological examination showed that these lobulations were correlated with the existence of well-defined cortical cords.

Histology of the gonads. The left ovary has a general appearance similar to that of the nine-day embryo. The cortex is very distinct, is thicker, and has much more massive cords of oogonia (Swift, '15). The zone of compact medullary tissue shows no apparent change. The spongy trabecular part has increased relatively.

Cortex of the left ovary. The cortex of the left ovaries examined shows the uniform character of development described by Swift ('15). The germinal epithelium is now reduced to a single layer of cubical to columnar cells. It is thicker occasionally between cortical cords. The cortex is thicker and less homogeneous in character due to the mitotic activity of primordial germ cells which has resulted in the formation of large cords of definitive oogonia, the cords of

the second proliferation. These cortical cords are composed of epithelial cells which become the definitive follicular cells and oogonia. The nuclei of the oogonia are very large, some being much larger than others. They are vesicular in appearance and show a marked condensation on the cytoplasm, the mitochondrial crescent. In some of the cords a few of the cells are in preleptotene stages of meiosis. The cortical cords are surrounded by connective-tissue stroma which isolates them from each other and from the medulla into which they extend. The primary tunica albuginea becomes less and less conspicuous in the older stages. The true ovarian albuginea is indicated between the cortical cords and the germinal epithelium by a few connective-tissue cells.

Cortex of the right ovary. The cortex of right ovaries at this stage of development shows variability. Of the nine embryos in this group studied histologically, four showed no indication of cortical rudiments, while five showed well-developed cortical cords in isolated areas. In the cases with no cortex there is only a flat germinal epithelium with single primordial germ cells occasionally included. The cells of the epithelium are usually flat to cubical, but occasionally columnar.

Of the specimens possessing cortical rudiments, E 140 of fifteen days' incubation shows a striking and exceptional development of cortex (figs. 3, 12, and 14). The right ovary in this case was shorter and wider than others of its age and was marked externally by rounded prominences. Histological examination showed that the lobulations are caused by the protrusion of large cords of oogonia. The distribution of this thick cortical layer is not continuous over the surface of the medulla, although it covers nearly the whole of the free surface of the gonad. Where the cortical cords are absent, the expanded medullary cords extend to the flat germinal epithelium. The germinal epithelium is also flat and well defined over the cortical cords. In a few spots it has cells elongated to a typical columnar type.

The cords of oogonia are massive, containing in certain cases over a hundred cells of the oogenetic line. A delicate layer of connective-tissue stroma separates the cords from the germinal epithelium, from each other and from the medulla. Neither primary nor definitive tunica albuginea is well developed. The cortex of this right ovary has a very different appearance from that of typical left ovarian cortex because of the great number of oogonia in a group without intervening strands of connective tissue. The oogonia themselves are practically identical with those of the left ovary. A few small epithelial cells are present among them.

Four other specimens showed smaller cortical cords of oogonia. These masses of oogonia are evidently developments of the sort of cortical rudiments observed in the earlier stages. Specimen E 494 of sixteen days' incubation which showed externally lobulations on the surface of the right ovary like specimen E 140, possessed correspondingly well-developed cortical cords, whereas specimen E 224, whose external surface was entirely smooth, showed no evidence of cortical rudiments (figs. 4 and 13).

Medulla of the left ovary. In the stage of development from fourteen days' incubation to hatching the medulla has increased in size due to the distention of additional medullary cords and the further distention of others. The texture consequently is more porous in appearance in sections than at nine days. The cavities of the cords extend throughout the medulla to the primary tunica albuginea. The distended cords distal to the hilus have cubical cells, while others are lined by squamous cells. The solid medullary cords which do not become distended gradually undergo, after approximately twelve days' incubation, a series of degenerative changes which sharply differentiate them. Round or oval masses of from four to twenty of the epithelial cells are bound together by a basement membrane and surrounded by a thin connective-tissue stroma. In most cases there is no lumen at the center of the cord. The cells become slightly larger than they were originally and the cytoplasm becomes

clear in sections, due to the formation of vacuoles by the dissolution of fat. The nuclei are round or oval and placed centrally or eccentrically. The nucleus shrinks very gradually, its karyoplasm stains more deeply and of the coarse masses of chromatin one or two stand out like nucleoli. These conspicuous cords are scattered throughout the medulla, but often form a band just within the cortex. Epithelial cells of these medullary cords are often found isolated in intertubular positions. In this condition they are conspicuously large and the cytoplasm is much vacuolated (fig. 10).

Such cords of clear cells have been observed frequently in previous studies of the gonads of the fowl. Waldeyer ('70) reported them first. Ganfani, Sonnenbrodt, and Poll observed them in the theca interna of ovarian follicles of the left ovary. Des Cilleuls ('12) and Firket ('14) called them interstitial tissue. Boring and Pearl ('17) distinguish the clear cells from other so-called 'interstitial cells' and (Pearl and Boring, '18) named them 'lutear' cells, ascribing to them an endocrine function in the ovary of the hen, homologous to the cells of the corpus luteum in mammals. Nonidez ('22 a, '22 b, '24) considers the term 'luteal' to be misleading and that the function of the cells is more likely trophic than endocrine. He says ('22 b):

Until their true function can be demonstrated, it seems more convenient to speak of the clusters as 'remnants of the sexual cords' since in both sexes they are derived from these structures, and we may refer to the cells themselves as the 'fat-laden cells' of the clusters.

Observations of Benoit ('23 c, '26 b), Poll and Fell ('23) confirm Nonidez' idea of their origin. Benoit calls them the 'glandular interstitial cells' and later 'L' cells or alveolar interstitial cells. In subsequent reference to these cells they will be called 'fat-laden cells,' following the usage of Nonidez.

Medulla of the right ovary. The slight increase in size of the right ovary in the stages preceding hatching is due chiefly to a greater number of distended medullary cords. The medulla of the right ovary is thinner than that of the left ovary, but varies both in its thickness and in texture in

different specimens and at different levels of the same ovary. The right medulla at its anterior extremity alongside of the postcaval vein is usually fibrous or poorly knit in texture and flatter than elsewhere. It is usually thickest at the level of its maximum width which occurs opposite the conjunction of the iliac veins. The right medulla varies in thickness from 0.14 mm. to 0.23 mm., whereas the solid part of the left medulla varies in thickness from 0.23 mm. to 0.3 mm. The right ovary of specimen E 239 is narrower and plumper, reaching a thickness of 0.39 mm. The correlation noted previously between the existence of cortical cords and more compact medullary tissue beneath them exists in these ovaries. Specimen E 140 of fifteen days' incubation and E 504 of eighteen days' incubation particularly illustrate this. Some of the ovaries have a more trabecular and fibrous appearance. Specimen E 239 of eighteen days' incubation is very spongy and is highly vascularized with large blood sinusoids filled with erythrocytes underlying the germinal epithelium.

The cellular constituents are the same as those in the left medulla. The number of compact medullary cords of cells with clear cytoplasm, the 'fat-laden cells,' possibly due to further degenerative changes in the right ovary has increased. They are conspicuous. In some embryos they are more numerous than in others. Some of the cords are cylindrical, while others appear elliptical in section. They contain from ten to twenty and more cells. As many as twelve such cords can be counted in an ovary. At this age, solid cords of primordial germ cells are more conspicuous in the medulla than at any previous stage of development. They are strikingly differentiated by their granular cytoplasm and large round vesicular nuclei. In many cases large cords contain both primordial germ cells and epithelial cells which have not degenerated. These latter cells show little or no vacuolation. The distended medullary cords are not well defined. The epithelial nature of the wall is often difficult to see because of the very squamous condition of the cells. The cavities appear frequently as irregular openings lined with

scattered nuclei and protoplasmic sheets. Medullary cells are found isolated and in phases of mitosis.

Primordial germ cells in the left medulla. The number and distribution of primordial germ cells vary. In some cases very few appear in the solid part of the medulla, while groups of two cells or isolated cells are scattered sparsely in the region of distended tubules close to the attachment of the gonad to the mesonephros. In another specimen such as E 246 of nineteen days' incubation primordial germ cells are very numerous throughout the left medulla. In this case as in others they are relatively less abundant than in the right medulla. Groups of two or three cells or cords of four to eight cells delimited by a basement membrane and stroma are found scattered in the medulla. Many of the primordial germ cells are isolated. Occasionally there are mixed cords with two or three primordial germ cells and four or more medullary cells surrounding them.

Primordial germ cells in the right medulla. In specimens of this age the primordial germ cells are especially numerous, and relatively more so in the right medulla than in the left. As many as ten to twenty are found in a single low-power field or from thirty to forty in the medulla. Some of the primordial germ cells are isolated. This is true particularly in the loose trabecular zone of the medulla. But the solid cords of primordial germ cells only, bound together by a basement membrane and strands of connective tissue are conspicuously abundant at this time. These cords are distributed throughout the medulla. Mitotic figures indicate that the increased number in groups is due to division. Groups of primordial germ cells are occasionally found loose inside of tubules or the elongated cavities of distended medullary cords. Very infrequently primordial germ cells are found in the epithelium. The primordial germ cells in the medulla are mostly in the resting stage. Some show a distinct mitochondrial crescent similar to the oogonia in the cortex. Some of the cells show preleptotene phases of meiosis. There is no lessened number of primordial germ cells in the medulla,

apparently, correlated with the presence of cortical cords. There are possible signs of alteration of primordial germ cells. Some appear to be shrunken. Some of the nuclei appear to have divided into one or two fragments. There is little evidence of any phagocytosis or aggregation of cells to aid in destructive processes.

Interstitial elements of the left medulla. Among the interstitial elements at this age of embryonic development is a very marked abundance of small round lymphocytes scattered profusely through the medulla and in the primary tunica albuginea. There are also hematopoietic foci with granular cells known as granulocytes.

Interstitial elements of the right medulla. Interdigitating strands of stroma with their characteristic connective-tissue nuclei are well defined between the cords. Hematopoietic foci extend through many sections and contain groups of ten to twenty granulocytes. Few, if any, of the small round 'primitive wandering cells' previously described are found after fourteen and fifteen days' incubation.

Chicks: First three weeks after hatching. The history of the left ovary subsequent to hatching has been described by d'Hollander ('04), Sonnenbrodt ('08), and others. The cortex increases greatly in size. According to Sonnenbrodt, mitosis among the oogonia stops at hatching and the growth of the oocytes follows. At three days after hatching, epithelial cells from the cortical layer begin to form the follicular layers around the oocytes, and by the eighth day the formation of the follicles is completed. After the first month the surface of the ovary becomes lobed and pebbled in appearance, due to the projection outward of the follicles containing oocytes. Sexual maturity is reached at the age of six months. The development of the left ovary will not be described further.

While the occurrence of a rudiment of the right ovary of variable nature and degree of development has been observed in the adult, the stages in the history of the rudiment after hatching have not been completely described previously

in the fowl. In the following study of the right rudiment after hatching the description of stages of development has been organized into three parts, namely, chicks of the first three weeks after hatching, chicks from three weeks after hatching to sexual maturity at six months, and adult hens.

For this stage, forty-one chicks were examined at the ages of 1 day, 3 days, 5 days, 8 days, 10 days, 12 days, 15 days, and 17 days after hatching. Serial sections were made of five of the specimens, namely, C 300 and C 800 of 3 days' age, C 405 of 8 days, C 312 of 10 days, and C 408 of 15 days.

Size and shape. The right ovary, in contrast to the left, at this time is retarded in development, but in all of the cases examined appeared as a small rudiment lying partly on the postcaval vein and partly on the right iliac vein. The rudiment was readily distinguished from the right mesonephros, alongside of which it lay. Its texture was more diffuse, but otherwise the gonad was quite similar to the stage previous to hatching. The length of the ovary as calculated from the five specimens sectioned ranged from 3.8 to 2.6 mm.

Cortex of the right ovary. Two of the five specimens examined histologically showed presence of cortex well developed, C 300 and C 800. Specimen C 408 showed a very small extent of columnar epithelium with primordial germ cells included.

In spots the germinal epithelium was columnar instead of flattened and in it were large oogonia or oocytes. At places beneath the columnar epithelium and also under the flattened epithelium were cords consisting of from four to ten oogonia only. In specimen C 800 there were large cortical cords containing thirty or forty oogonia (fig. 10). Probable development of follicles was indicated in one instance. In specimen C 800 there were several cords of epithelial cells homologous to those epithelial cells in the left ovary which had become differentiated as follicular or granulosa cells. In one of these there was an oocyte in the center (fig. 10, *cor.cd*). The others consisted only of columnar epithelial cells with unvacuolated granular cytoplasm. The nuclei were peripherally placed,

in which respect they differed from the clusters of 'fat-laden cells' of the medulla, whose nuclei tended to be grouped centripetally. The nuclei of these follicular cells resembled those of the follicular cells of the left ovary in every detail. The karyoplasm stained darkly, and there were large granules. The shape was oval or round and the size noticeably larger than the round nuclei of the 'fat-laden cells.' Benoit ('26 b) considers such cells which he calls type C to transform into alveolar interstitial cells (type L) similar to those formed from remnants of medullary cords of the first proliferation.

Medulla of the right ovary. The texture and composition of the right medulla is practically the same as noted for the stages just preceding hatching. The outlines of the distended medullary cords have become more irregular. The clusters of 'fat-laden cells' have become very numerous and are conspicuous because of their clear vacuolated cytoplasm. In these the amount of cytoplasm has increased and the nuclei are pushed toward the center of the cord. The numbers of clusters of 'fat-laden cells' is relatively more than at earlier stages. Intermediate stages exist in which medullary cords containing both epithelial cells and primordial germ cells are transforming into clusters of 'fat-laden cells.' The few epithelial cells which have been isolated from the cords grow to very large size and are round.

Primordial germ cells in the right medulla. Primordial germ cells are readily observable in all of the specimens examined. They are usually found isolated or in pairs, but occasionally there are groups of four to eight. Some of the sections showed only a few primordial germ cells or oogonia, but in sections taken at different levels it was common to find from six to twenty. Very often single primordial germ cells or groups of two or four were loose in the cavities of distended medullary cords.

Chicks: Three weeks to six months. For the stage between three weeks after hatching and sexual maturity at six months, fifty-three specimens were examined at intervals of two

weeks. Serial sections were made of the right ovaries of
twelve of these.

The right ovary is no longer visible as a distinctly de-
limited body, but lies on the ventral and lateral surface of
the postcaval vein in the form of wrinkled strands of tissue
which cannot easily be distinguished from the tissue of the
mesonephros and the postcaval vein. The gonad tissue may
extend a slight distance onto the ventral surface of the right
iliac vein.

Sectioning shows that the length of the rudiment varies
from 2.2 mm. to 6.8 mm. in length. The measurements show
a variation in the length, but also a gradual increase in
length with increase in age as follows:

Specimen			Specimen		
C 414,	22 days,	2.2 mm.	C 501,	90 days,	4.4 mm.
C 831,	35 days,	4.3 mm.	C 502,	90 days,	6.7 mm.
C 422,	36 days,	3.5 mm.	C 505,	104 days,	5.3 mm.
C 430,	63 days,	4.5 mm.	C 508,	118 days,	4.5 mm.
C 433,	77 days,	4.4 mm.	C 510,	146 days,	6.8 mm.
C 500,	90 days,	5.8 mm.	C 517,	174 days,	5.5 mm.

The texture of the gonad tissue is much more fibrous as a
whole than at any stage previous. There is, however, con-
siderable variability in its compactness which is correlated
with its width and thickness, for as the tissue lengthens it
thins out.

Width of the gonad at different levels and in different
chicks varies from 0.3 mm. to 1.2 mm. The usual width where
the gonad attains its maximum size is about 0.8 mm. The
thickness varies from 0.05 mm. to 0.4 mm. For the most
of its extent the gonad is less than 0.15 mm. thick. Greater
thickness is usually due to a more spongy and loose condi-
tion. Specimen C 414, 22 days old, contains a very scant
amount of tissue which is in the form of loose strands spread
widely and thinly on the ventral surface of the postcaval
vein. The gonad usually consists of a thick mass adjacent
to the mesonephros, which tapers laterally to thinness on
the surface of the postcaval vein.

A striking exception to the prevailing condition of the rudiment is that shown in specimen C 505, 104 days old, in which there is a thick cortex with well-developed follicles (fig. 16). In this case the gonad is over 1 mm. wide and 0.5 mm. thick.

As a rule, it is difficult to diagnose the presence of cortical rudiments without sectioning. In the older chicks the whole gonad is often overlaid by a thick layer of connective tissue which is continuous with that of the surface layers of the postcaval vein. The presence of cortex, however, can usually be detected on external examination because the masses of follicles are more opaque and whiter than the medullary tissue. From external examination it is evident that follicles may occur in spots sporadically at different regions of the rudiment. Sometimes there are several such spots in a single rudiment.

Cortex of the right ovary. The germinal epithelium consists of a single layer of flattened cells, ordinarily. In specimen C 505, 104 days, which has well-differentiated cortex over nearly the whole surface the germinal epithelium is for the most part columnar and one or two cells thick. A few strands of definitive tunica albuginea underlie it.

Cortex exists in three others of the sectioned specimens, C 430, 63 days, C 510, 146 days, and C 517, 174 days. In specimen C 430 most of the rudiment is medullary tissue only, but at one spot there are several oocytes surrounded by follicular cells. There are also groups of follicle cells by themselves. In specimen C 517, 6 months old, there is one group of many follicles which extends over a millimeter in length, and then at some distance from this group is another small group of oocytes in follicles. Clusters of follicular cells alone are also present among the follicles. Specimen C 510 has one small group of follicles.

The cortex in specimen C 505 is typically ovarian (fig. 16). It extends over most of the surface of the gonad, which is over a millimeter wide, for nearly its entire length. Its thickness depends on the size of the follicles. The follicles

vary in diameter from 0.05 mm. to 0.3 mm. As many as nine follicles are present in a single section. The more mature follicles have penetrated into the medulla, and at no place is the cortex sharply distinguishable from the medulla.

Medulla of the right ovary. As previously mentioned, the medulla is more compact where it adjoins the mesonephros. For the most part it is very fibrous and trabecular. The distended medullary cords have indefinite walls and the clusters of 'fat-laden cells' and isolated cells abut into them. Cords of clear 'fat-laden cells' are conspicuously abundant throughout the medulla. Some of these cords whose degeneration evidently began earlier than that of others show large vacuoles and nuclei which have contracted and shrunk. In others the nuclei are large and round. A few isolated medullary cells are found. Occasionally a mixed cord with a shrunken primordial germ cell surrounded by 'fat-laden cells' is observed.

Primordial germ cells in the right medulla. Primordial germ cells are found very rarely. They are usually isolated and in rather shrunken condition. Occasionally, a group of two or three together is found. It is a noteworthy fact that in all of the specimens of this stage examined histologically there is practically a complete absence of primordial germ cells in the medulla of the right ovary. Not more than one or two are ever found in a section, and most sections show none whatever. The few that are found usually consist of a very shrunken nucleus and very little of the typical granular cytoplasm. The probable fate of the primordial germ cells appears to be a disappearance due to gradual atrophy. There was some indicating that the nucleus at times breaks down into smaller fragments.

Interstitial elements of the right ovary. Interstitial elements of the right ovary which should be noted are occasional groups of granulocytes in hematopoietic foci. In some specimens large cords of dark-staining granular cells appear, which have the appearance of an involuting body. Fibrous connective-tissue cells were present, but none gave appearance of differentiation for secretion.

Adult hens: One year and six months old. At this age fifteen specimens were examined. Serial sections were made of two of the rudimentary right ovaries. Hens at this state of maturity show three types of development of the right gonad. In the majority of the cases (eleven specimens) the right gonad is a rudiment of poorly defined tissue lying on the ventral face of the postcaval vein lateral to the mesonephros. In two exceptional cases, C 601 (fig. 5) and C 680, there is an unmistakable ovary somewhat smaller in size than the normal left ovary, but composed of large follicles. The third type of development occurs apparently in a few instances. The rudiment is similar to the first type described, except for the presence of small lumps of cortical tissue containing four or five follicles. These lumps may be scattered at some distance apart along the vein. Survey of adult hens thus leads to the conclusion that the rudiment of the right ovary which persists has a capacity for development which ranges from somewhat normal cortex to medulla only.

Cortex of the right ovary. The cortex which develops on the right side appears identical with that of the left ovary, although it is less in size. In specimen C 680 the left ovary was 3 cm. long and 1 cm. wide, while the right ovary was 2 cm. long and 0.7 cm. wide. In specimen C 601 the left ovary was 2.8 cm. long and 1.5 cm. wide, while the right ovary was 1 cm. long and 0.5 cm. wide. Specimen C 603, which showed two small groups of follicles spaced widely apart in the region of the rudiment, was sectioned. The oocytes surrounded by their follicular layers were identical to similar follicles in the adult left ovary (fig. 17, 18).

Medulla of the right ovary. In specimen C 600, which had no rudiments of cortex, the medullary tissue lay as a thin sheet on the ventral surface of the postcaval vein for a distance of 7.7 mm. In specimen C 603, which had two small groups of follicles, the medullary tissue extended through 9.2 mm. The cellular composition of the medulla is the same as that of the preceding stage. Distended medullary cords are present. Clusters of 'fat-laden cells' with clear yellow-

ish cytoplasm are conspicuous. Some of these are isolated and round in shape.

Primordial germ cells in the medulla of the right ovary. There is no evidence of primordial germ cells in the right ovary except for an occasional shrunken nucleus whose identification is doubtful.

History of the mesonephros and mesonephric duct

In the literature it is commonly stated that the mesonephric bodies and mesonephric ducts of the female degenerate shortly after hatching (Firket, '20). In the present investigation, along with the study of the ovaries, a chronological examination was made of the mesonephroi and the mesonephric ducts. Distinct remnants of both the right mesonephros and the right mesonephric duct were found to persist in females to sexual maturity and in adult hens a year and a half old. The later stages of the left mesonephros and mesonephric duct were not studied in detail, but apparently their fate is the same as the right.

In the chicks examined just after hatching the mesonephroi are easily recognized. They are reduced in size, but easily distinguishable from the three-lobed metanephroi. In later stages the left mesonephros is concealed by the greatly enlarged left ovary. The right mesonephros, however, was located as a small body in all of the specimens. In chicks over three weeks old the mesonephros is separated from the metanephros and lies as a small strand of tissue on the lateral wall of the postcaval vein. The right ovary is adjacent to the mesonephros medially (compare figs. 15, 16, 17). From the mesonephros the mesonephric duct can be traced posteriorly. It is thread-like and small in diameter, but has a patent lumen. The duct crosses the surface of the metanephros and lies adjacent to the metanephric duct, alongside of which it enters the cloaca.

Histological preparations were made of the mesonephros at different ages. At three weeks after hatching, the bulk of the body consists of collecting tubules. Toward the median

side large glomeruli are conspicuous and numerous. The rete cords, situated between the mesonephros and the medulla of the ovary, are not well defined. At thirty-five days after hatching, the body of the mesonephros consists mostly of a mass of convoluted tubules, the epoophoron which is the homologue of the epididymis of the male. Renal corpuscles are scarce. The mass of rete cords is now conspicuous, consisting of anastomosing cords with open lumina. In specimens of 63 days, 77 days (fig. 15), and 147 days (6 months), after hatching, the tubules of the epoophoron have larger lumina, the epithelial walls of the tubules are reduced, and the rete tubules are very conspicuous. In the adult hen the right ovary is some distance from the right mesonephros, but long rete tubules connect the two (fig. 17). The mesonephros consists of a reduced number of convoluted tubules and in some respects has the appearance of an involuting body.

From the specimens examined it is shown that there exists in the adult hen, in relation to the right rudimentary ovary by means of the rete cords, epoophoron, and mesonephric duct a basis for a complete viable system of testis ducts (figs. 1, 3, 4, 6, 12, 13, 14, and 18).

Summary statement of results

A primary result of this investigation has been to demonstrate that, contrary to the views of many previous investigators, a rudiment of the right ovary persists not only through embryonic life, but remains after hatching up to adult life as a mass of tissue of variable composition.

In 61 per cent of the fifty-six specimens examined histologically at various ages the rudiment consists of medullary tissue only (thirty-four cases). In 39 per cent of the specimens (twenty-two cases) cortical rudiments were present.

The development of medullary and cortical tissue was followed chronologically from nine days' incubation to a year and a half after hatching.

Considering, first, cases lacking cortical tissue, it is essential to follow the fate of the medullary cords, the fate of the primordial germ cells, and the fate of the interstitial elements.

At nine days' incubation there is a medulla filled compactly with the medullary cords formed in the first proliferation of sexual cords at six days' incubation. The germinal epithelium consists of flattened cells which are quiescent. There is no secondary proliferation of sexual cords homologous to the cortical cords which normally develop in the left ovary at approximately eleven days' incubation. The right ovary in these cases is therefore homologous to a testis in an embryological sense.

The medullary cords follow two courses of development. Some of them, even before nine days' incubation, begin to form lumina in their centers, following which they become more and more distended until they are scarcely recognizable as derivatives of epithelium. As the lumina become large, they form irregular cavities which extend throughout the gonad, and the epithelial cells become very much flattened. The result is that the medulla in adults appears to be of a loose reticular nature. It becomes less and less compact, so that, subsequent to the stage of three weeks after hatching, the loose strands of tissue spread out thinly on the postcaval vein, causing the rudiment to be longer than at any time in its early development. In the adult a thick layer of connective tissue continuous with the adventitious layers of the postcaval vein grows over the surface of the medulla, which makes it difficult to distinguish as a gonad. Other medulla cords assume a peculiar differentiation. After fourteen days' incubation groups of four to twelve and more epithelial cells, remnants of the medullary cords, are conspicuously separated into groups by a basement membrane and surrounding layers of connective tissue. The cytoplasm of the cells becomes very clear, due to infiltration of fat, upon the dissolution of which in fixation large vacuolations are left. The vacuoles increase in size so that the cords of cells are much expanded

in the tissue of adult gonads. The nuclei of these fat-laden cells stain more darkly than ordinary epithelial cells. They are round at first and tend to be pushed toward the center of the cords. In some of the adult tissue the nuclei have shrunk to irregular masses. Such remnants of the medullary cords as here described are very conspicuous in the rudiment of the right gonad in adult hens.

Primordial germ cells, at the time the gonad is first formed, are scattered throughout the mesenchyme, which is later included in the medulla. When the first proliferation of sexual cords occurs at six days' incubation, additional primordial germ cells are carried into the medulla along with the epithelial cells of the medullary cords. Still other primordial germ cells may migrate into the medulla from the germinal epithelium by their power of amoeboid movement. At first the primordial germ cells are quiescent. After nine days' incubation, however, mitotic activity is seen among them occasionally, and in the stages just before and just after hatching there are nests or groups of two to eight oogonia which are the result of such activity. The transformation of primordial germ cells into oogonia and oocytes occurs more slowly in the medulla than in the cortex of the left ovary. All of the primordial germ cells do not become oogonia at the same time, but some of them have the mitochondrial crescent characteristic of oogonia after nine days' incubation. Some of the oogonia show preleptotene stages of meiosis, but very few, if any, develop into oocytes. By three weeks after hatching, the number of primordial germ cells, which previously was relatively great, is much reduced. Up to three weeks after hatching, oogonia are conspicuously numerous in the medulla. As many as ten to twenty can be counted in most of the sections. In the specimens examined after three weeks subsequent to hatching there was a striking reduction in the number of oogonia leading to complete disappearance ultimately. The few still persisting at three weeks showed signs of degeneration chiefly by shrinkage and atrophy. In earlier stages, such as eighteen days' incu-

bation, occasional oogonia were seen with shrunken nuclei. Apparently, the fate of the primordial germ cells in the medulla is atrophy and disappearance a short time after hatching.

Interstitial elements of the medulla in the early stages of development consist of connective-tissue cells derived from the embryonic mesenchyme of the gonad, primitive wandering cells, lymphocytes, and granulocytes, and isolated medullary cells derived from the medullary cords of the first proliferation of sexual cords. In later stages of development and in the adult the connective-tissue cells give no indication of differentiation for secretory function. Granulocytes are often found late in developmental stages and in adults. No secretory function is ascribed to them. Large round isolated medullary cells are found throughout development and in the adult.

The history of right gonads in which cortical rudiments are found and in which follicles appear in the adult in general parallels that of the normal left ovary. At nine days' incubation there is a medulla homologous to that of the left ovary composed of medullary cords formed by the first proliferation of sexual cords at six days' incubation. The germinal epithelium, however, instead of being reduced to a single layer of flattened cells, has in such cases several layers of columnar epithelial cells, among which are numerous primordial germ cells. While the amount of such epithelium is small and sporadic in occurrence over the surface of the gonad, it is identical in appearance with that of the left ovary.

At eleven days' incubation in gonads which possess these rudiments a second proliferation of sexual cords occurs giving rise to cortex. In some of the embryos very large cortical cords composed of oogonia and epithelial cells are formed. This is contrary to the condition in the left ovary where strands of connective-tissue stroma separate the cells into smaller cords. The cortex in the right ovary is discontinuous, and it may occur at several spots widely separated on the

surface of the ovary. The cortical cords are constricted from the germinal epithelium after twelve days' incubation. The behavior of the cortical rudiments after hatching is for a time similar to that of the cortex of the left ovary. The oocytes enlarge, and at three days after hatching epithelial cells form follicular layers about them. In the later stages of development and in the adult hen large follicles resembling in every respect those of the left ovary may be occasionally found. Also cords or tubules of columnar follicular cells without oocytes are found. The nuclei of these cells are round or elongated and filled with large granules; the cytoplasm is finely granular. There is some evidence that such cells may become isolated and interstitial in position. Such cells are not found in rudimentary right ovaries without cortical rudiments.

The fate of the medulla associated with cortical rudiments on the right side is similar to that of rudiments on the right side in which medulla alone persists, and also the medulla of the left ovary. Remnants of the medullary cords persist as clusters of fat-laden cells in the medulla. Isolated epithelial cells from the medullary cords are also found.

DISCUSSION

The facts concluded from this investigation are a basis for the interpretation of a number of problems. The fact of the persistence of a rudiment of the right gonad was logically to be inferred from the exceptional cases in which right ovaries bearing follicles were found in adult birds. Chronological investigation of the right gonad gives support to the hypothesis that the type of gonad tissue, whether ovarian or testicular, which develops on the site of the right gonad following ovariectomy depends on the embryological composition of the rudiment (Lillie, '27). Willier ('27) discusses this possibility in connection with the problem of asymmetrical growth capacities in the right and left embryonic ovaries.

The persistence of remnants of the medullary cords as the chief components of the right rudimentary ovary in the great majority of adult hens furnishes an embryological basis for the testis-like hypertrophied body which occurs on the site of the right ovary following ovariotomy. The medullary cords of the ovary are homologous with the definitive sexual or seminiferous cords of the testis. When the inhibitory influence of the left ovary is removed under the experimental conditions of ovariotomy, it is to be expected that such medullary cords might come to resemble testicular elements. Benoit ('24, '26 a) explains the occurrence of seminiferous tubules in the right testis-like gonads in his cases of ovariotomized females by the assumption of transformation of medullary cords. Where cortical rudiments as well as a medulla are found in the right ovary, ovotestis or ovary may be expected to develop (Finlay, '25; Domm, '27).

Thus the various possible developments on the site of the right ovary, both under normal and experimental conditions, seem to be determined by the embryonic composition of the rudiment. However, the extent to which the development capacity of the rudiment is realized may be partially dependent on other factors. In the embryos of nine days' incubation, 39 per cent of the right ovaries had cortical rudiments, while in the adult hens studied only 20 per cent have cortical rudiments. While the apparent reduction of cortical rudiments in later stages may be due to some differential factor in the collecting of the specimens, there is a probability that the left ovary may inhibit the development of the cortex of right ovaries just as it inhibits the medullary components of the right gonad. In any case the nature of whatever kind of structure develops from the right rudiment will be influenced by the persistence or non-persistence of primordial germ cells. It is possible that, under the experimental conditions of ovariotomy, an environment favorable to persistence of primordial germ cells would be provided, so that in chicks ovariotomized at a time when primordial germ cells are relatively numerous in the medulla of the

right rudimentary ovary, that is, before the fourth week after hatching, primordial germ cells might persist and undergo progressive development. Ovariotomy performed after the third week after hatching, when the primordial germ cells have undergone regression, would not be expected to yield such results. Whether or not persisting primordial germ cells undergo a differentiation into spermatozoa, the continued existence of the rete, epoophoron and mesonephric duct affords a basis for a complete viable system of testis ducts.

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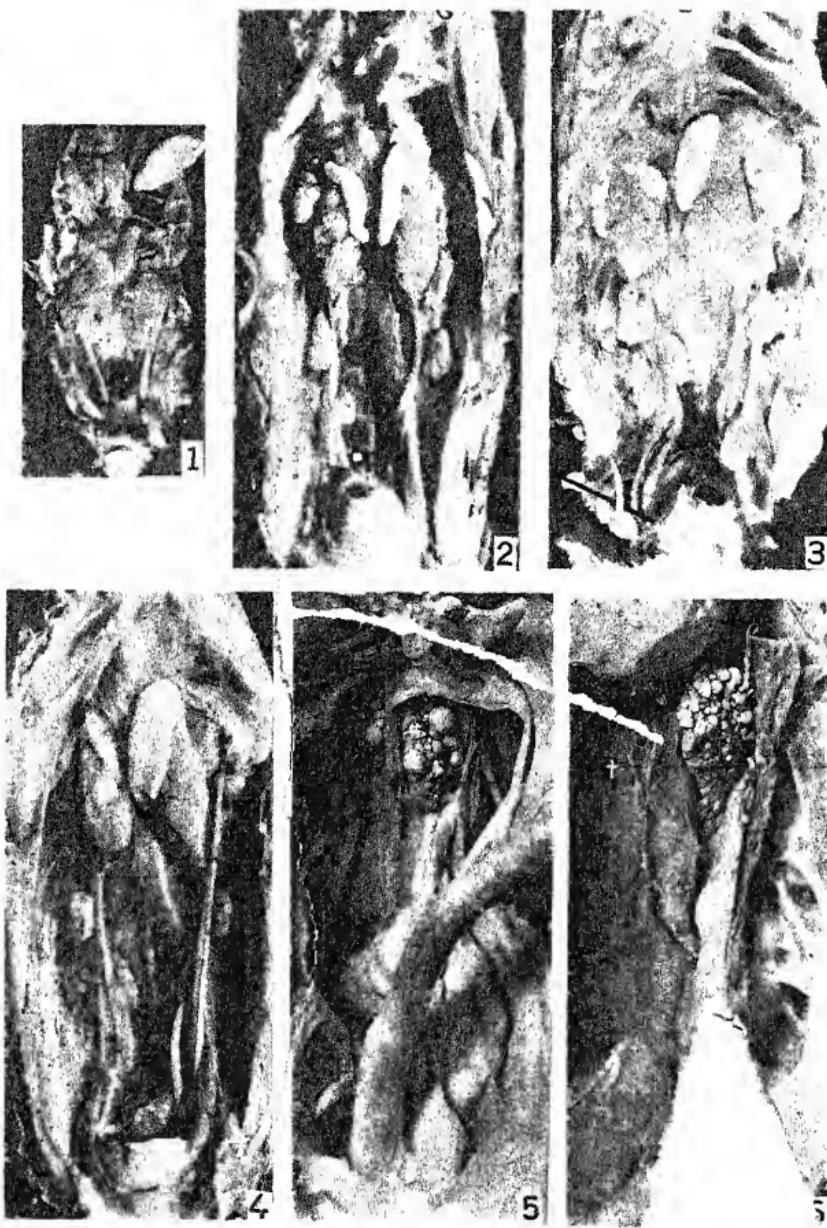
PLATE 1

1 Photograph of dissection of ten-day ♀ embryo, E 124. Right and left ovaries are shown on medial faces of mesonephroi. Right ovary, 2 mm. long; left ovary, 2.5 mm. long. Right and left müllerian ducts are conspicuous and both extend 7 mm. to the anterior extremities of the mesonephroi.

2 Photograph of dissection of fifteen-day ♂ embryo, E 471. The right testis shows a characteristic flexure similar to that of the right ovary of the same age. Lobes of the metanephroi are visible lateral to the mesonephric ducts. Medial to them are the ureters. The müllerian ducts have disappeared entirely.

3 Photograph of dissection of fifteen-day ♀ embryo, E 140. Right ovary, 2 mm. long; left ovary, 3.5 mm. long. The right ovary shows on its surface exceptional cortical lobulations. The left müllerian duct extends from the cloaca to the anterior extremity of the left metanephros. Its proximal end is dilated. The right müllerian duct has become reduced to 6 mm. The left müllerian duct is 17 mm. long. The mesonephric ducts and ureters are medial in position.

4 Photograph of dissection of sixteen-day ♀ embryo, E 224. Similar to fifteen-day ♀ embryo, E 140. The right ovary shows no lobulations.

5 Photograph of dissection of adult hen, eighteen months old, C 601. An exceptional development of follicles is shown to the right of the dorsal mesentery and wholly separated from the left ovary. Right ovary, 10 mm. long, 5 mm. wide; left ovary, 28 mm. long, 15 mm. wide.

6 Photograph of dissection of adult hen, eighteen months old, C 650. † indicates the normal condition of the right rudimentary ovary, viz., a thin tissue lying on the postcaval vein adjacent to the remnant of the right mesonephros.

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PLATE 2

7 Drawing of a portion of the cortex and medulla of the left ovary of embryo E 302 of nine days' incubation. Initial magnification, 1100 times. Shows normal condition of left germinal epithelium with included primordial germ cells (*oög.*). *g.ep.*, germinal epithelium; *p.t.a.*, primary tunica albuginea; *cor.cd.*, cortical cord; *m.c.*, medullary cord cells.

8 Drawing of portion of right ovary of embryo E 437 of nine days' incubation. Initial magnification, 1500 times. Shows the usual flattened condition of the germinal epithelium of the right ovary. The exceptional occurrence of two primordial germ cells among the flattened epithelial cells is shown. *g.ep.*, germinal epithelium; *pr.*, primordial germ cell; *pr.mi.*, primordial germ cell in mitosis; *m.c.*, medullary cord cells.

·9 Drawing of portion of the cortex and medulla of the right ovary of embryo E 302 of nine days' incubation, showing exceptional development of cortical rudiments. Initial magnification, 1100 times. *g.ep.*, germinal epithelium; *oög.*, oogonium; *m.cd.*, medullary cord; *pr.mi.*, primordial germ cell in mitosis; *m.cd.d.*, distended medullary cord.

10 Drawing of portion of the right ovary of chick C 800, three days after hatching. Initial magnification, 1100 times. Shows cortical rudiments composed of follicles enclosing oocytes. *g.ep.*, germinal epithelium; *cor.cd.*, cortical cord with oocyte; *c.*, cord of oogonia derived from medullary primordial cells; *oöc.*, oocyte surrounded by follicular cells; *fol.cd.*, cord of follicular cells derived from epithelial cells of the cortex; *m.c.i.*, isolated medullary cord cell; *m.cd.d.*, distended medullary cord; *m.c.f.*, cluster of 'fat-laden cells,' remnant of medullary cord of first proliferation; *pr.i.*, isolated primordial germ cell.

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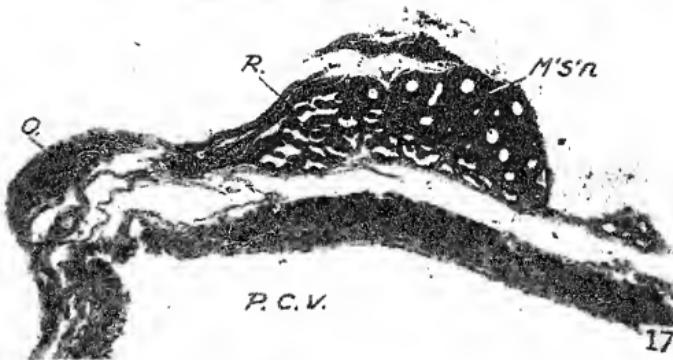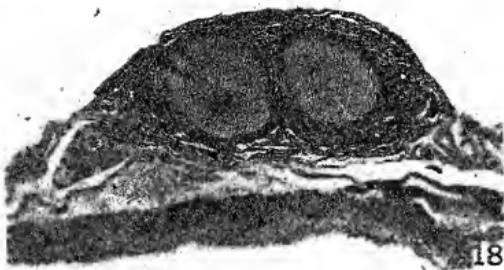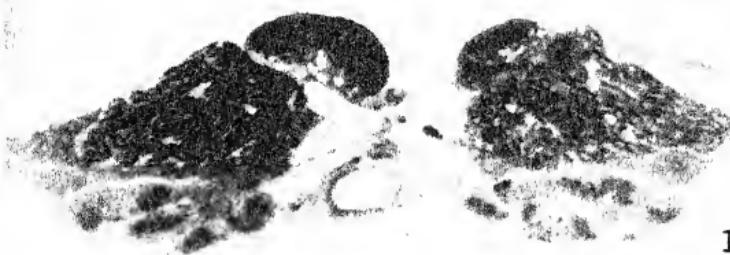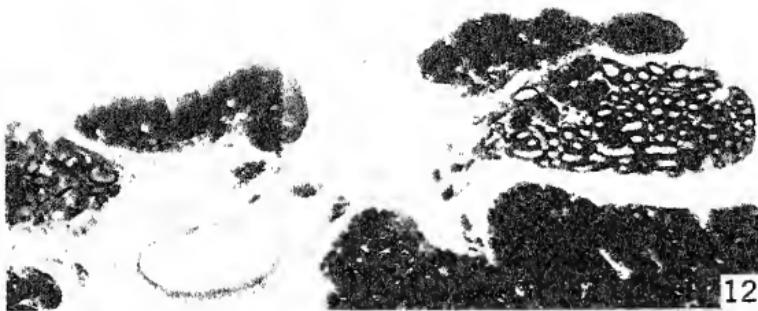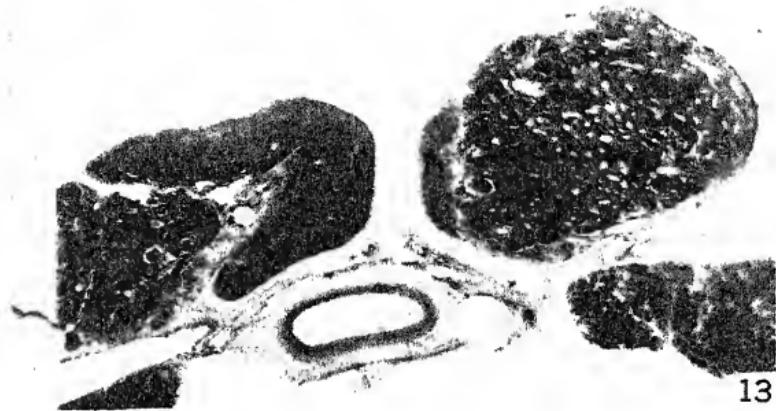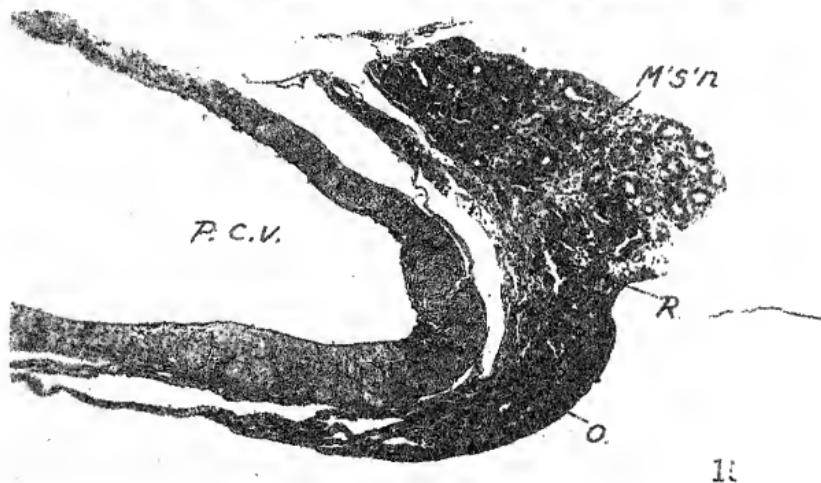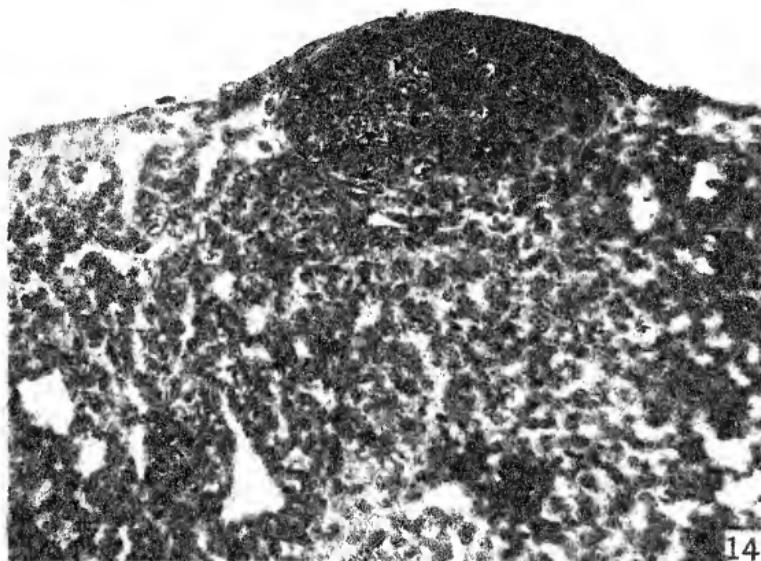
PRENATAL GROWTH OF SWINE[1]

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SEVEN FIGURES

AUTHOR'S ABSTRACT

Fetuses from forty-three gravid uteruses from sows of known breeding dates, as well as from 448 uteruses with unknown breeding dates, were studied. Growth curves are given for weight and length of fetus and for weight of fetal membranes. The weight of the fetus first reaches that of the fetal membranes between the sixtieth and seventieth days of pregnancy. Degenerate fetuses were found in 3.68 per cent of the cases. They were found at all stages of gestation. Size of litter was found to decrease from 11.4 at the twentieth day to 6.8 at the 110th day. Also, the calculated per cent of ova lost up to each ten-day stage tends to increase as gestation advances. Crowding was found to be an important factor, but probably not the only factor, in causing degeneration. Genetic factors were probably responsible for part of the resorbing fetuses.

In the study of the normal fetuses, significant correlations were found between fetus length and weight of fetal membranes, as well as between fetus weight and weight of fetal membranes. Lower correlations, but probably significant, were found between total distance (spacing) between fetuses in the uterus and weight of fetal membranes. Correlations between size of fetus and total distance between fetuses were very low. As in the case of the degenerates, crowding has an important relationship to size of fetus, but is probably not the only factor involved.

A study of birth records of swine has shown that there is much variation in size of the offspring at birth. It seems to be generally assumed that variations, other than qualitative characters, such as color, are due more largely to the environment in utero than to the genetic make-up of the different fetuses. An attempt is made to separate some of the in utero environmental factors which influence the development of the pig embryo from the genetic factors. This resolves itself into a study of the positions and sizes of embryos and fetuses in the same uterus, comparison with the size of the fetal membranes, and comparison with the amount of space available.

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MATERIAL STUDIED

gnant uternses and their contents were studied at pack-
ases at Chicago, Illinois, Madison, Wisconsin, and
ille, Maryland. Most of these uteruses were from sows
niscellaneous herds with unknown breeding dates. To
material to use as a guide to the ages of untimed
s, fifty sows and gilts of the Poland-China, Duroc-
-, and Chester-White breeds were mated by the writer,
lled at definite dates after breeding. Of these animals,
were found to be pregnant. Records of three gravid
ses from sows of known breeding dates killed at Belts-
vere added. The fetuses from these forty-three gravid
ses form the basis for the estimation of age of the
nder.

ACKNOWLEDGMENTS

STUDY OF FETUSES OF KNOWN AGES

In order to estimate the ages of the fetuses obtained from untimed packing-house material, it was necessary to study some of known ages. No data of this nature have been presented since the limited amount given in Keibel's Normentafeln in 1897. The tables given by Stöckli ('22) and in Strangeways' Veterinary Anatomy ('92) go back principally to Gurlt, whose publications appeared in the early part of the last century, and, so far as the writer can determine, are not available in this country. Types, as well as methods of feeding swine, have changed so greatly since then that it seemed essential to obtain some data from present-day swine. Furthermore, the above-mentioned tables are not definite enough to be of the most use.

Through the courtesy of the Animal Husbandry Department, fifty-three head of sows and gilts were placed at the writer's disposal. These were kept at the Serum Plant and were self-fed corn and tankage. Conditions did not permit hand feeding, yet it seems logical to believe that these data are comparable to those obtained from the untimed material obtained at packing-houses. The writer made daily observations and mated the animals as often as estrum was observed. He also kept all breeding, slaughtering, and other records. Several of the gilts were never observed in heat and were finally sold open. Some of the others proved to be non-pregnant, one contracted pneumonia and died, and forty were pregnant when slaughtered. In addition to the above, four gilts were bred at the Beltsville farm of the U. S. Department of Agriculture and examined by Dr. H. C. McPhee at the time of slaughter. Three of these were pregnant, and he kindly forwarded copies of the records. An attempt was made to divide the slaughtering dates so that the time after breeding of each female would be at ten-day intervals. The earliest was twenty days after breeding. It was impossible to have the sows at Beltsville killed at exactly twenty or thirty days after breeding. Of the remainder, it was necessary for various reasons to vary the time one to two days

each in three cases.   The other thirty-seven head are on exactly ten-day intervals.

Through the courtesy of Oscar Mayer & Co., it was possible to obtain the uteruses as soon as the carcasses were opened. All measurements, weights, and other records of the uteruses and their contents were taken as soon as possible on the killing floor.

The following procedure was followed in obtaining the data. The uterus was placed in a measuring box, and each ovary
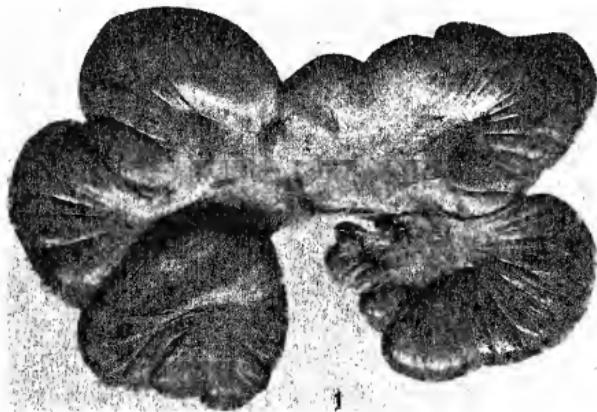
Fig. 1 Gravid uterus of gilt.   The left ovary had been removed before breeding and adhesions formed which held the left end of the uterus in a twisted shape.   In all other respects this photograph is typical of gravid uteruses as obtained for study on the killing-floor.

removed, weighed, and the corpora lutea counted.   Each broad ligament was then cut away from its attachment to the corresponding horn of the uterus, and the uterus and contents weighed (figs. 1, 2, and 3).   Then the uterus was returned to the measuring box, and the length of each horn measured in centimeters.   Each horn was split longitudinally, by means of an enterotome, on a line opposite the line of attachment of the broad ligament.   The location of each embryo or fetus, its sex when determinable, and whether it was normal or degenerated were noted.   The distance between each two

fetuses and between each end of the horn and the nearest fetus was next obtained in millimeters. The points used were the placental attachments of the umbilical cords. The length of each fetus was taken from crown to rump by means of calipers. After measurement, the umbilical cord was cut from the body of the fetus, and the fetus was weighed in grams. After each fetus was removed, its fetal membranes were carefully stripped from the uterine wall, and, after drainage of the excess of moisture, were weighed in grams. After all of the contents of the uterus were removed, the empty uterus was weighed. Figure 2 shows the measuring box and an unopened uterus, with ovaries and broad ligaments removed.
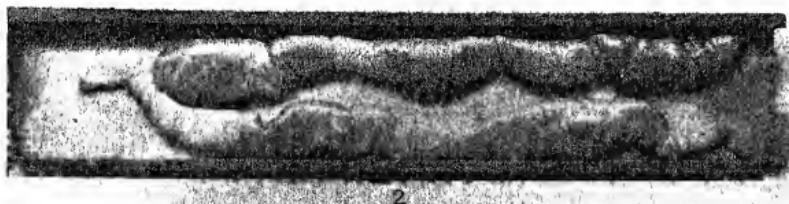
Fig. 2 Gravid uterus of gilt, after removal of the ovaries and the broad ligament. This shows the measuring box used in this study.

The data on number and sex of fetuses[2] of different ages together with the weights and measurements and weights of fetal membranes are presented in table 1.

The relation of length of fetus to age is shown graphically in figure 4, and of weight to age, in figure 5. The weights of the fetal membranes at known ages show a decrease toward the end of the gestation period (fig. 5), but this may be due to fluctuation of the sampling, for when larger numbers are used, from fetuses of estimated ages, the apparent dip in the curve almost disappears, as is also shown in figure 5.

[2] While it is usual to refer to the fetus in its earlier stages as an embryo, in this paper, for convenience, the term fetus is used throughout the develop-
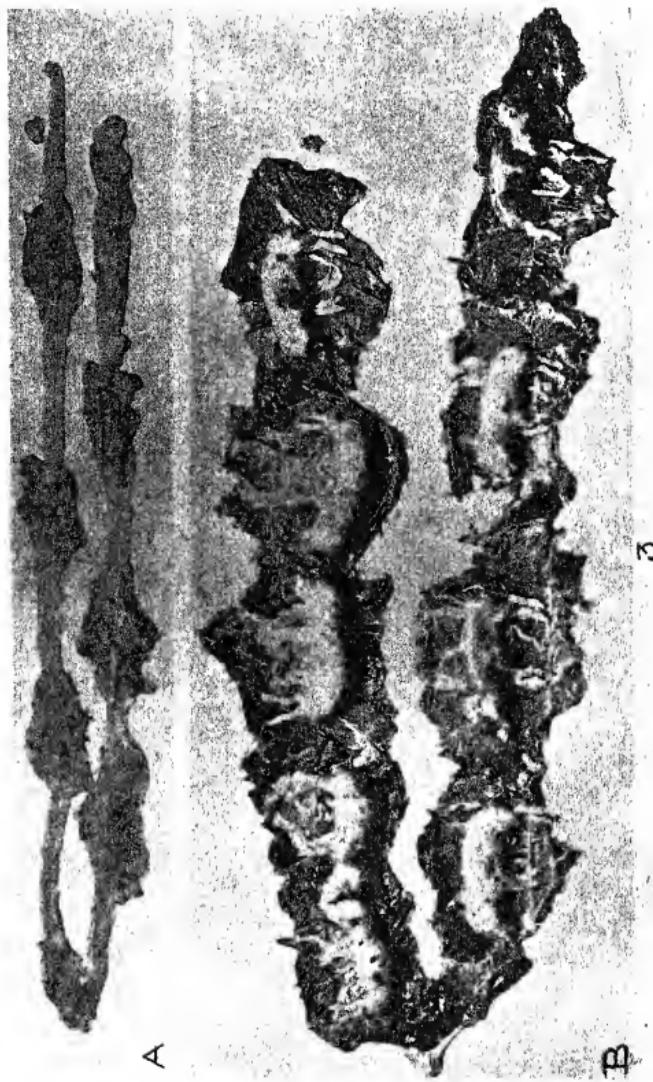
Fig. 3 Gravid uteruses of gilts after breeding. The broad ligaments have been removed, and each horn has been split longitudinally. These gilts had been semispayed before breeding, but except for one ovary having been removed from each, the uteruses are typical. A) Uterus from gilt twenty-six days after breeding. B) Uterus from gilt ninety days after breeding.

The weight of fetus first equals that of the fetal membranes between the sixtieth and the seventieth day after breeding, as is also indicated in figure 5.

It is not the purpose of this paper to discuss the question of increment of growth or growth cycles during intra-uterine life of the fetus. The primary object of the inclusion of the data from timed material is to furnish a basis for estimating the age of the material of unknown age. Special attention is
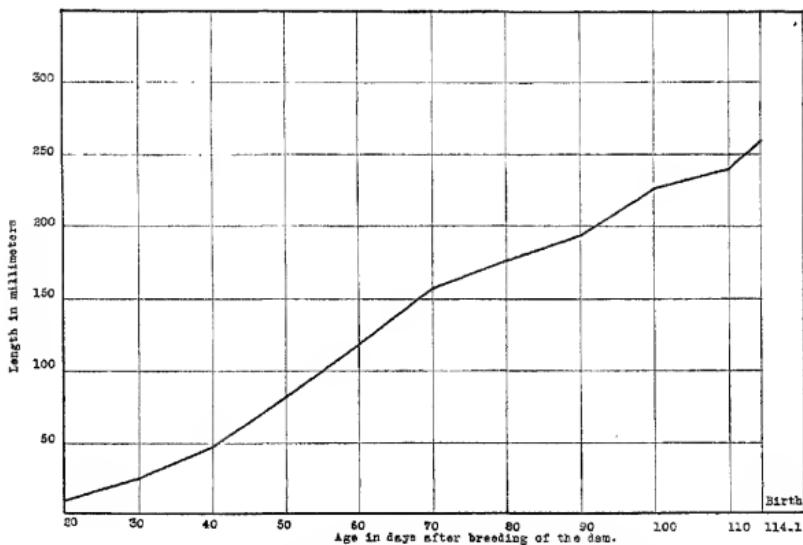
Fig. 4 Curve showing relationship of length of fetus to age.

called to the wide variation in size of the apparently normal fetuses in the same uterus, and different uteruses of the same stage of pregnancy. The sizes in the different age groups of the latter half of the gestation period overlap to a large extent, but those of the younger ages do not. For this reason, it seems reasonable to believe that, in estimating the age of untimed fetuses, the average of the normal individuals of the litter should be used rather than actual measurements of any one individual. Length is commonly employed to desig-

nate the size of pig fetuses, so is used in our estimates of age. The writer realizes that, even by using these averages from timed material to estimate the age, some of the known-age fetuses themselves would be estimated somewhat older or somewhat younger than they actually are, but it appears to be the best 'yard-stick' we have.

Fig. 5 Curves showing relationship of weight of fetus to age, and of weight of fetal membranes to age.

OCCURRENCE OF DEGENERATE EMBRYOS AND FETUSES

Among 3967 fetuses examined in apparently healthy uteruses, 146, or 3.68 per cent, were found to be in various stages of degeneration. The distribution according to age is seen in table 2. These figures show a somewhat lower percentage of degenerates than was found by Hammond ('21) and by Corner ('23) in swine.

Table 2 demonstrates that the largest per cent occurred during the earliest stage examined, but almost as large per

TABLE I. FETUSES OF DEFINITELY KNOWN AGES

n = normal; d = degenerated (resorbing), not included in averages

Column headers (repeated per panel):
Uterus Number | Length of Fetus (mm.) | Weight of Fetus (gm.) | Appearance | Uterine Membr. Wt. (gm.) | Fetus Number

20 DAY FETUSES, **22 DAY FETUSES**, **26 DAY FETUSES**, **27 DAY FETUSES**, **29 DAY FETUSES**, **30 DAY FETUSES**, **32 DAY FETUSES**, **40 DAY FETUSES**, **50 DAY FETUSES**, **60 DAY FETUSES**, **70 DAY FETUSES**, **71 DAY FETUSES**, **80 DAY FETUSES**, **90 DAY FETUSES**, **100 DAY FETUSES**, **110 DAY FETUSES**

TABLE I. SUMMARY

Age in Days (Uterus Number)	Total Fetuses	Normal Fetuses				Degenerating Fetuses			
		Number and Sex	Average Length (mm.)	Average Weight (gm.)	Av. Wt. of Membranes (gm.)	Number and Sex	Average Length (mm.)	Average Weight (gm.)	Av. Wt. of Membranes (gm.)

¹These two litters are recorded as of different days but the actual interval between them is only 6½ hours   ²Average of eight   ³Average of six   ⁴Average of three   ⁵One fetus

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age. The writer realizes that, even by using these averages
from timed material to estimate the age, some of the known-
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Fig. 5   Curves showing relationship of weight of fetus to age, and of weight
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during the earliest stage examined, but almost as large per

cents occurred at other stages, particularly the last two. The appearance of the degenerate fetuses showed that many of them were in a process of resorption. Undoubtedly, most of those occurring in the earliest stages had completely disappeared by the time the later stages were reached. Occasionally, degenerate fetuses remain until the sow farrows and are expelled during normal parturition. A study of the number of fetuses present at each stage of pregnancy gives results as shown in table 3. From this it may be seen that

TABLE 2

Appearance of all fetuses in both right and left horns

ESTIMATED AGE	TOTAL FETUSES	NORMAL	DEGENERATE	DEGENERATE
Days	*Number*	*Number*	*Number*	*Per cent*
20	148	137	11	7.43
30	270	259	11	4.25
40	283	269	14	4.95
50	528	513	15	2.84
60	690	679	11	1.59
70	658	638	20	3.04
80	466	441	25	5.36
90	347	341	6	1.73
100	194	181	13	6.70
110	383	363	20	5.22
Totals and average	3967	3821	146	3.68

the number of fetuses decreased from an average of 11.4 at the twenty-day stage to 6.8 at the 110-day stage. However, the number of corpora lutea was also decreased as pregnancy advanced, although to a much smaller extent. Whether this was an actual decrease or due to some error in determining the number is not known. It is possible that some corpora lutea of estrum persisted and were included in the counts of the earlier stages. No histologic study was made to determine this point. It may be assumed that the difference between the average number of corpora lutea at each stage and the number of fetuses present represents ova that have been lost by not

being fertilized or by death and resorption later. Table 3
also shows the per cent lost at each stage. This is also shown
in figure 6. The fitted line denotes a distinct upward trend
in the per cent of ova lost at each stage as gestation advances.

Four and twenty-three hundredths per cent of the fetuses
located in the right horns and 3.13 per cent of those in the left
horns were degenerated (fig. 4). There is no significant dif-
ference in the total number of fetuses in the right and left
horns. Of the 146 degenerates observed, 57.53 per cent were
in right and 42.46 per cent in left horns. If it is assumed

TABLE 3

*Estimated loss of ova at each stage of gestation, as shown by comparison of
corpora lutea with fetuses*

ESTIMATED AGE	NUMBER OF UTERUSES	NUMBER OF CORPORA LUTEA		NUMBER OF FETUSES		OVA UNACCOUNTED FOR	
		Total	Average	Total	Average	Average	Per cent
Days							
20	13	186	14.3	148	11.4–	2.9	20.28
30	30	361	12.0	270	9.0	3.0	25.00
40	31	340	11.0–	269	8.7–	2.3	20.09
50	55	628	11.4	500	9.1–	2.3	20.17
60	81	889	11.0–	680	8.0	3.0	27.27
70	78	902	11.5	629	8.0	3.5	30.43
80	60	605	10.1–	454	7.5	2.6	25.74
90	46	439	9.5	347	7.8–	1.7	17.89
100	27	256	9.5–	193	7.1	2.4	25.26
110	54	513	9.5	371	6.8	2.7	28.42

that they are as likely to occur in one horn as the other, the
probable error of the deviation from expectation is ± 4.07.
The deviation from the expected 50 per cent in each horn
is 7.53. This is only 1.8 times the probable error. This
indicates that the difference is probably not significant. The
numbers of fetuses in the right and left horns tend to become
equal even when there are unequal numbers of corpora lutea
in the two ovaries. Experimental proof of this has been
presented in a recent paper by the author ('26). In this ex-
periment sows were semispayed, bred, and then slaughtered
during pregnancy. Even though all the eggs came from one

ovary, the numbers of fetuses became equalized in the two horns.

Degenerate fetuses have been found in all possible numerical positions from the bifurcation. The tabulation in table 5 shows that in each of the first five positions from the bifurcation the percentage of occurrence is about the same, but rapidly increases in the positions farther removed.

Each horn was then taken as a unit and classified as to the number of fetuses present. The per cent of horns containing degenerate fetuses was determined in each group. It was found that 8.66 per cent of all the horns contained one or
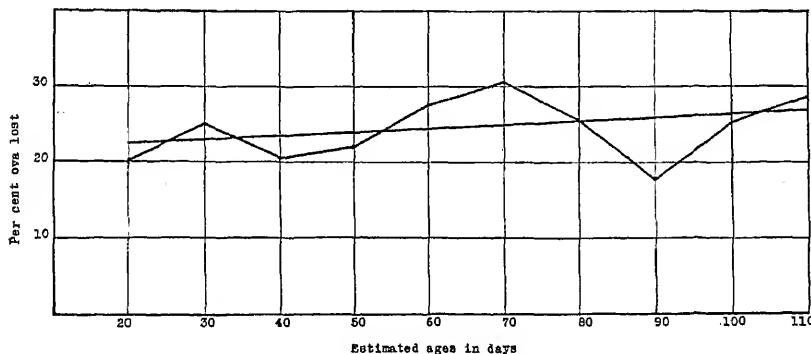
Fig. 6   Per cents of ova missing at each estimated age, with the fitted line.

more degenerate fetuses. Table 6 gives the per cents according to the number of fetuses present.

This indicates that there is a decided tendency for the degenerates to occur more frequently when there are larger numbers in the same horn, yet they occur even when only one fetus is present and there can be no crowding.

The average space per fetus was determined for each horn of each uterus whose fetuses were estimated to be ninety-five days of age or older. Each horn was then classified and all the horns were grouped in a frequency table. The number of horns containing one or more degenerate fetuses was next determined for each group of the frequency table. The results are given in table 7.

TABLE 4

Occurrence of degenerates in right and left horns of the uterus

ESTIMATED AGE	RIGHT HORN			LEFT HORN		
	Total	Normal	Degenerate	Total	Normal	Degenerate
Days						
20	74	69	5	74	68	6
30	136	131	5	134	128	6
40	149	140	9	134	129	5
50	260	251	9	268	262	6
60	351	342	9	339	337	2
70	325	315	10	333	323	10
80	241	227	14	225	214	11
90	166	162	4	181	179	2
100	96	88	8	98	93	5
110	190	179	11	193	184	9
Totals	1988	1904	84	1979	1917	62
Per cent			4.23			3.13

TABLE 5

POSITIONAL OCCURRENCE OF DEGENERATE FETUSES

ESTIMATED AGES IN DAYS

POSITION	20		30		40		50		60		70		80		90		100		110		TOTAL FETUSES	TOTAL DEGENERATE	PER CENT DEGENERATE
	TOTAL	DEGENERATE	TOTAL	DEGENERATE	TOTAL	DEGENERATE	TOTAL	DEGENERATE	TOTAL	DEGENERATE	TOTAL	DEGENERATE	TOTAL	DEGENERATE	TOTAL	DEGENERATE	TOTAL	DEGENERATE	TOTAL	DEGENERATE			
1R,L	26	1	59	3	64	3	115	4	167	4	163	3	123	6	91	2	53	3	111	5	972	34	3.5
2R,L	26	1	57	3	64	4	112	5	163	0	157	3	119	6	89	0	49	2	106	5	942	29	3.1
3R,L	26	2	52	1	59	4	109	2	146	2	136	2	105	5	79	0	43	3	83	5	838	26	3.1
4R,L	23	1	42	1	47	3	87	4	104	1	95	1	65	3	54	0	32	2	57	4	606	20	3.3
5R,L	19	2	30	0	26	0	64	0	59	2	60	1	35	2	22	2	14	2	19	1	348	12	3.5
6R,L	14	2	13	1	16	0	27	0	28	1	25	2	11	0	8	0	3	1	6	0	151	7	4.6
7R,L	10	2	8	1	7	0	6	0	13	0	10	1	3	1	2	0	0	0	1	0	60	4	6.7
8R,L	2	0	4	0	0	0	5	0	5	0	3	1	3	1	2	0	0	0	0	0	24	2	8.3
9R,L	2	0	3	0	0	0	3	0	3	0	3	3	1	0	0	0	0	0	0	0	15	3	20.0
10R,L	0	0	1	0	0	0	0	0	1	0	3	1	1	1	0	0	0	0	0	0	6	2	33.3
11R,L	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	2	1	50.0
12R,L	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	100.0
13R,L	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	100.0

[1] Number stands for position from bifurcation; R, right horn; L, left horn

TABLE 6

Distribution of degenerate fetuses in the horns according to the number of fetuses

NUMBER OF FETUSES IN HORN	TOTAL NUMBER OF HORNS	NUMBER OF HORNS WITH DEGENERATE FETUSES	PER CENTS OF HORNS WITH DEGENERATE FETUSES
1	29	1	3.4
2	104	5	4.8
3	232	13	5.6
4	257	15	5.8
5	198	18	9.1
6	89	18	22.2
7	36	6	16.6
8	9	2	22.2
9	9	0	0.0
10	3	3	100.0
11	2	2	100.0
12	0	0	0.0
13	1	1	100.0

TABLE 7

Per cents of horns which contain degenerate fetuses when classified according to average space. Estimated age of all fetuses in this table ninety-five days and older

AVERAGE SPACE PER FETUS	TOTAL HORNS	HORNS CONTAINING DEGENERATES	PER CENTS OF HORNS CONTAINING DEGENERATES
cm.	*Number*	*Number*	
15– 19	1	1	100.00
20– 24	6	4	66.66
25– 29	11	3	27.2
30– 34	29	5	17.2
35– 39	26	1	3.8
40– 44	25	1	4.0
45– 49	16	0	0
50– 54	12	0	0
55– 59	7	0	0
60– 64	9	0	0
65– 69	5	0	0
70– 74	5	0	0
75– 79	2	0	0
80– 84	1	0	0
85– 89	1	0	0
90– 94	1	0	0
....	...	...	....
135–139	1	0	0

This demonstrates that when the average amount of space is extremely small, a very large percentage of the horns contain degenerate fetuses. No horns in this age group had degenerate fetuses when the average space per fetus was over 45 cm. These results are probably most largely dependent on the number of fetuses present, which has been discussed above.

VARIATION IN SIZE OF NORMAL FETUSES

The variation in size is not limited to a comparison of normal and degenerate embryos and fetuses. As noted above, the sizes of the normal fetuses of the ages beyond the middle of the gestation period show such extreme variation that the frequency distributions of the different age groups overlap. A study of the different correlations should throw some light on the probable causes.

The fetus derives its nourishment from the mother by means of the fetal membranes. Does the size of the fetal membranes directly influence the size of the fetus? The only satisfactory measure of the fetal membranes of the pig is weight. Length of fetus was correlated with weight of fetal membranes in each estimated age group, using only the normal individuals. The lowest coefficient of correlation was .3178 ± .0240 in the 65- to 75-day group, and the highest was .6478 ± .0266 in the 25- to 35-day group. Similar correlations, using weight of fetus instead of length, give higher coefficients. The lowest is .3770 ± .0317 at 75 to 85 days, and the highest is .5914 ± .0195 at 45 to 55 days. These show that there is a direct relationship of fetus size to size of the fetal membranes.

If the fetuses and their membranes are crowded closely together, it seems that the development would be retarded, as Ibsen ('23 a, '23 b) has reported in guinea-pigs. The amount of space on each side of the fetus was added together and called total distance. This is, obviously, more than the average space, but should be of value from a comparative standpoint. The weight of the fetal membranes was correlated

with total distance in each of the estimated age groups except the youngest. The lowest coefficient was .2379 ± .0351 in the 85- to 95-day group, and the highest was .4880 ± .0382 in the 95- to 105-day group. This shows that the amount of crowding influences the size of the placenta to some extent. However, the coefficients of correlation are not much above the border-line of significance. No high correlations have been found in this material such as those reported by Ibsen in guinea-pigs.

Correlation of length of fetus with total distance and of weight of fetus with total distance did not give any markedly significant correlations. The small amount of correlation must come indirectly from the effect on the fetal membranes. All of the coefficients of correlation are shown in table 8 and plotted against age in figure 7. Owing to the relatively small number of fetuses of any known age, it was not possible to determine the relation of weight to number of fetuses.

The records of the fetuses of the three oldest groups, ninety days and over, were classified according to numerical position from the bifurcation, and their weights were averaged. Obviously, it would be misleading to combine different age groups to get averages unless the total number of fetuses were the same in each age group. Any influence due to numerical position would manifest itself toward the end of pregnancy. For this reason the above-mentioned age groups were selected. The average weights for each position are given in table 9 for the three age groups. This shows that there is no constant ranking, and so numerical position of itself does not determine the size.

Fig. 7 Curves showing the coefficients of correlation at different stages of gestation, with the fitted straight line for each curve. The coefficients of correlation marked 1 to 7, inclusive, should each be preceded by a decimal point.

TABLE 8

COEFFICIENTS OF CORRELATION ON PRENATAL GROWTH					
EST. AGE DAYS	LENGTH-WEIGHT	LENGTH-FETAL MEMBRANE	LENGTH-TOTAL DISTANCE	WEIGHT-FETAL MEMBRANE	WEIGHT-TOTAL DISTANCE	FETAL MEM-BRANE-TOTAL DISTANCE
30	.8352 ± .0127	.6478 ± .0266	.0746 ± .0418	.5486 ± .0321	.0709 ± .0419	.2787 ± .0423
40	.9247 ± .0061	.3446 ± .0371	.0370 ± .0416	.4093 ± .0350	.1012 ± .0416	.4879 ± .0321
50	.9207 ± .0046	.5527 ± .0208	.0532 ± .0299	.5914 ± .0195	.1029 ± .0296	.2380 ± .0283
60	.8936 ± .0052	.4019 ± .0217	.1924 ± .0249	.4395 ± .0209	.1934 ± .0293	.2466 ± .0243
70	.8051 ± .0094	.3178 ± .0240	.0648 ± .0266	.4146 ± .3286	.0905 ± .0265	.3318 ± .0380
80	.7608 ± .0141	.5526 ± .2231	.3018 ± .2919	.3770 ± .0317	.1856 ± .0310	.3797 ± .0275
90	.7504 ± .0123	.3390 ± .0330	.0859 ± .0370	.4380 ± .0301	.0425 ± .0380	.2379 ± .0351
100	.8501 ± .0139	.3942 ± .0423	.1554 ± .0489	.4272 ± .0410	.1478 ± .0490	.4880 ± .0382
110	.8319 ± .1093	.4365 ± .0287	.2330 ± .0337	.4808 ± .0273	.1338 ± .0350	.3693 ± .0308

TABLE 9

Average fetus weights according to numerical position from bifurcation

| NUMERICAL POSITION	90-DAY FETUSES		100-DAY FETUSES		110-DAY FETUSES	
	Average weights	Rank	Average weights	Rank	Average weights	Rank
	Grams		Grams		Grams	
1	449	6	566	2	734	6
2	425	8	533	6	735	5
3	446	7	598	1	713	7
4	453	5	546	4	750	(3 + 4)
5	456	4	563	3	750	(3 + 4)
6	491	3	543	5	906	1
7	516	2	...	...	781	2
8	562	1	...	...	...	...

Fig. 7 Curves showing the coefficients of correlation at different stages of gestation, with the fitted straight line for each curve. The coefficients of correlation marked 1 to 7, inclusive, should each be preceded by a decimal point.

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COEFFICIENTS OF CORRELATION ON PRENATAL GROWTH

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5	456	4	563	3	750	(3 + 4)
6	491	3	543	5	906	1
7	516	2	...	...	781	2
8	562	1	...	...	...	...

SUMMARY AND DISCUSSION

Fetuses from forty-three gravid uteruses of known breeding dates were studied. The growth curves show that length increases at a fairly uniform rate throughout the gestation period, while weight increases much more rapidly during the last twenty days than earlier. The weight of the fetus first reaches that of the fetal membranes between the sixtieth and seventieth days of the gestation period. There is much variation in the sizes of the fetuses of the same age, especially during the last half of pregnancy. For this reason it seems logical in any study in which untimed pig fetuses are used, to estimate the age by use of the average of all of the normal individuals of the litter rather than by the measurement of any particular individual.

In addition to the timed material, the fetuses from 448 uteruses with unknown breeding dates were also studied. These uteruses contained 3967 fetuses, of which 146, or 3.68 per cent, were found to be in various stages of degeneration. These were found in each age group, with the largest per cent in the youngest group. However, there was no regularity from the youngest to the oldest. Due to the process of resorption, probably all the fetuses which became degenerate at early ages would become resorbed before the end of the gestation period. This is shown by the fact that litter size decreases from 11.4 at the twenty-day stage to 6.8 at the 110-day stage. Also the calculated per cent of ova lost up to each age tends to increase as gestation advances. The degenerate fetuses in the older material were probably of recent occurrence in most cases. However, there was so much variation in the degree of resorption that it was impossible to estimate the length of time which had elapsed since the death of the fetus. It seems logical to believe that the death of the fetus is as likely to occur at one age as another.

Fetuses in the first five numerical positions from the bifurcation included about the same per cent of degenerates in each. The other positions had much higher percentages of degenerates. This is probably due in part to the large number

of fetuses present, which in turn reduced the amount of space available for each fetus. It was found that both a large number of fetuses per horn and small average space was accompanied by a marked increase in number of horns containing degenerates. This would imply that crowding was the cause of degeneration of the fetus. Crowding is without doubt an important factor involved, particularly during the latter half of the gestation period. It is probably, however, not the only factor involved. A degenerate fetus was found in one case where it was the only fetus present in one of the horns of the uterus. There was no crowding in this case. Other degenerates were found which showed no sign of crowding. Degenerates were most numerous during the earliest stages studied, even when the fetal membranes had not extended over the full area between two adjacent fetuses. Furthermore, when a fetus dies and resorption begins, the fetal membranes of the normal fetuses next to it tend to occupy its space. This would give the appearance of crowding, even though crowding had nothing to do with the death of the fetus. Inherent genetic differences in the germ plasm of the fetuses themselves probably are responsible for part of these resorbing fetuses, as suggested by Corner ('23). It is even possible that all of the degenerates were the result of genetic factors. The fact that as many as nine normal fetuses have been observed in a single horn with no degenerates would tend to support this hypothesis that degeneration is dependent primarily on genetic factors rather than overcrowding. It may be that fetuses which are inferior genetically are particularly susceptible to the effect of crowding.

In the study of the normal fetuses, significant correlations were found between fetus length and weight of fetal membranes. Comparable correlations were found between fetus weight and weight of fetal membranes. This shows that there is a direct relationship between the growth of the placenta and of the fetus. This is only natural, and it is surprising that the correlations are not even higher.

Lower correlations, but probably significant, were found between total distance between fetuses and weight of fetal membranes. This shows that crowding has some effect in retarding the development of the fetal membranes. This being the case, since, as has been shown, size of fetus is correlated with fetal membranes, the size of the fetus should also show positive correlations with total distance between fetuses. The correlations were positive, but very low. As in the case of the degenerates, crowding has some effect on the size of the normals. Besides this the low coefficient of correlation shows that there must be other factors operating to increase the variability. These are probably genetic. It seems logical to infer that some of the fetuses carrying poor combinations of factors escaped death during the early part of pregnancy, but lacked the ability to develop into large vigorous pigs.

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ADDENDUM

Since this manuscript has gone to press, Ibsen has published his complete data on "Prenatal growth in guinea-pigs," which demonstrate the effect of crowding on fetus size in that species.

IBSEN, H. L. 1928 Prenatal growth in guinea-pigs, with special reference to environmental factors affecting weight at birth. Jour. Exp. Zoöl., vol. 51, pp. 51–91.

GROWTH OF PARAMECIA IN PURE CULTURES OF PATHOGENIC BACTERIA AND IN THE PRESENCE OF SOLUBLE PRODUCTS OF SUCH BACTERIA [1]

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SIX CHARTS

AUTHOR'S ABSTRACT

Virulent hay-infusion cultures of Bacillus pyocyaneus are toxic to pure-line races of three species of paramecia, but these races may acquire a tolerance for this toxic agent. Races with acquired tolerance have been grown for long periods of time in toxic, pure cultures of B. pyocyaneus by means of the daily-isolation culture method, and here the average division rate is as high as, or higher than, in the chance-mixed bacterial cultures in which these protozoa are usually maintained in the laboratory. The tolerance is lost, however, when the paramecia are removed from the toxic cultures and grown for a number of generations in cultures of non-toxic bacteria.

The toxic agent that is lethal to paramecia is probably the soluble toxin of B. pyocyaneus. The investigation shows that the agent is soluble and either thermolabile or volatile. It also shows that all deleterious substances, other than the soluble toxin, known to be produced in cultures of this bacillus, are non-lethal to paramecia.

Hay-infusion cultures of Bacillus enteritidis were lethal to paramecia. All attempts to develop tolerance in paramecia for the toxic agent in these cultures failed.

Under the experimental conditions that prevailed, diphtheria toxin was found to have no appreciable effect upon the division rate or death rate in three species of paramecia.

INTRODUCTION

In this initial investigation of the effect of pathogenic bacteria and bacterial products upon protozoa the first problem at hand was that of selecting materials with which to work. Paramecium was chosen because of the ease with which it may be grown under laboratory conditions. Moreover, it can be grown in pure cultures of bacteria, as has been shown by Hargitt and Fray ('17) and Phillips ('22). Bacillus pyocyaneus and Bacillus enteritidis were selected because they are pathogenic and are able to grow luxuriantly in the hay-infusion media that are commonly used for paramecia. It was also the desire to select some standardized toxin whose effect upon paramecia could be studied. A number of toxins

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the University of Missouri.

are available for such a study, including those of bacterial
origin as well as those produced by higher plants and animals.
In the present investigation, however, only one standard
toxin, that of the diphtheria bacillus, has been used.

The investigation has been exploratory in its nature. In
the main it was the intention to study the effect of these
pathogenic bacteria and bacterial products upon paramecia
and to determine the extent to which races of paramecia are
able to adapt themselves to the deleterious effects of these
agents. It was also hoped that the study might eventually
yield data of significance in connection with certain funda-
mental questions in pathology and immunology. It was felt
that the study might incidentally offer an opportunity to
extend the technique of Hargitt and Fray ('17) and of Phil-
lips ('22) in methods of growing paramecia in pure cultures
of bacteria. There was also the opportunity to continue the
search, initiated by these investigators, for a species of bac-
teria in pure cultures of which paramecia may be grown
successfully.

The present investigation has been in progress since the
spring of 1924. Most of the work has been carried on at the
Marine Biological Laboratory, Woods Hole, Massachusetts,
and at the Zoölogical Laboratory of the University of Mis-
souri. The study has been made possible largely through
the encouragement and help of Prof. W. C. Curtis, of the
University of Missouri, and through the facilities of his
laboratory. To Prof. L. L. Woodruff, of Yale University,
thanks are also due for his interest during the summer of
1925. For assistance and for materials and apparatus thanks
are due Dr. Mazyck P. Ravenel, of the Department of Medical
Bacteriology, and to Prof. W. J. Robbins, of the Department
of Botany, both of the University of Missouri.

MATERIALS AND METHODS

Materials

Bacillus pyocyaneus Gessard (Pseudomonas aeriginosa
Schroter Migula) and Bacillus enteritidis Gaertner (Salmon-

ella enteritidis Gaertner) were selected as the pathogenic bacteria to be used in this investigation, but only the former proved to be favorable. To make the work more comprehensive, experiments were conducted with three species of paramecia, Paramecium aurelia and P. calkinsi, from Prof. L. L. Woodruff's pedigreed stocks maintained at Yale University, and P. caudatum from wild strains. In each experiment the controls and experimental animals were obtained from a single pure line established for the purpose.

Diphtheria toxin was selected as a soluble toxin to be used in the study, because of its availability and standardization. Time has not permitted the extension of the study to other standard toxins.

Further explanations of the materials used appear in connection with the accounts of particular experiments.

Culture media

The standard hay infusions used in this study were made by adding 10 grams of chopped timothy hay to a liter of water and boiling for five minutes. This is referred to throughout this report as '0.2 per cent standard hay infusion.' Ordinary distilled water was used in some experiments. In others tap-water was used. The kind of water used is specified for each experiment. Enough hay infusion was made each time to last throughout the experiment and this was placed in 100-cc. Erlenmeyer flasks, that were then plugged with cotton and sterilized in an Arnold sterilizer by the intermittent method. The pH of each infusion was obtained after sterilization and recorded for each experiment.

Method of washing the paramecia bacteria-free

To grow paramecia in pure cultures of bacteria it is, obviously, necessary to free the animals from miscellaneous bacteria attached to their surface. In the present work animals were sterilized by a method which differed very slightly from those devised by Hargitt and Fray and by Phillips. Each

animal to be freed from bacteria was transferred by means of sterile pipettes through eight sterile wash waters. The first two wash waters were contained in culture slides in a sterile Petri dish. These slides had deep cylindrical cavities each containing approximately 0.5 cc. of liquid, which gave the animal a greater volume of sterile medium and thus facilitated sterilization. The last six waters through which each animal was passed were contained in watch crystals, as in the work of Phillips. Animals were transferred in each case under the low power of a binocular, and in each transfer the lid of the Petri dish was raised barely enough to permit the entrance of the capillary pipette. The large end of each pipette was plugged with cotton to prevent contamination from the rubber bulbs which were not sterilized. As a fresh sterile pipette was used for each transfer from one sterile water to another, it was necessary to have them on hand in large numbers. Ground-glass-stoppered specimen jars were used as containers, each containing as many as twenty-five pipettes. These were sterilized in hot air. These and other standard forms of technique used by bacteriologists were applied throughout the work.

Efficiency of this method

Tables 1 and 2 show the efficiency of the foregoing method of sterilizing paramecia. Nineteen individuals of P. caudatum and fourteen of P. aurelia were washed, and in each case a record was made to show, a) whether the water in which the animal was washed was sterile (column 7); b) whether the last two wash waters remained sterile after the washing (columns 2 and 3); c) whether each washed animal when introduced into 0.5 cc. of sterile hay water continued to live more than twenty-four hours (columns 4 and 5), and, d) whether the sterile water into which the washed animals were introduced remained sterile (column 6). The sterility of this water was determined in each case by placing a portion of it on the surface of an agar slant and incubating the tube at 22°C. for two weeks. In this manner a total of thirty-three

animals were washed in the order shown in tables 1 and 2, and no cases were omitted.

From the data in tables 1 and 2 it may be concluded that a paramecium can be completely sterilized by the method above described. In table 1, animals 5, 6, 9, 10, 13, 14, 15, and

TABLE 1

Efficiency of washing nineteen individuals of Paramecium caudatum. The animals are listed in the order in which they were washed, and no cases are omitted

(1) NO.	(2) 7TH WASH	(3) 8TH WASH	(4) CONDITION OF ANIMAL 24 HOURS AFTER WASHING	(5) CONDITION OF ANIMAL 48 HOURS AFTER WASHING	(6) CONDITION OF WATER 48 HOURS AFTER WASHING	(7) CONTROL WASH WATER
1	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
2	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
3	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
4	Sterile	Sterile	(Animal died in 7th wash)			
5	Sterile	Sterile	Dead	Dead	Sterile	Sterile
6	Sterile	Sterile	Dead	Dead	Sterile	Sterile
7	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
8	Sterile	Sterile ·	Dead	Dead	Contaminated	Sterile
9	Sterile	Contaminated	Dead	Dead	Sterile	Sterile
10	Sterile	Sterile	Dead	Dead	Sterile	Sterile
11	Contaminated	Contaminated	Dead	Dead	Contaminated	Sterile
12	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
13	Sterile	Sterile	Dead	Dead	Sterile	Sterile
14	Sterile	Sterile	Dead	Dead	Sterile	Sterile
15	Sterile	Sterile	Dead	Dead	Sterile	Sterile
16	Sterile	Contaminated	Dead	Dead	Sterile	Sterile
17	Contaminated	Sterile	One alive	One alive	Contaminated	Sterile
18	Sterile	Contaminated	One alive	One alive	Contaminated	Sterile
19	Sterile	Sterile	One alive	Two alive	Contaminated	Sterile

16 were successfully sterilized. In table 2, animals 1, 3, 6, 7, 10, 11, 12, and 14 were successfully sterilized. Out of a total of thirty-three animals washed, sixteen were rendered completely bacteria-free.

The data make it necessary to conclude, however, that the method cannot be relied upon to successfully sterilize every animal washed. Animals 1, 2, 3, 4, 7, 8, 11, 12, 17, 18, and 19

in table 1, and 2, 4, 5, 8, 9, and 13 in table 2 were not sterile, for in each case the hay water in which the animal died showed bacterial contamination.

Incidentally, the results shown in the above study throw some light upon the length of time paramecia will live in sterile media. Combining the results obtained with P. cau-

TABLE 2

Efficiency of washing fourteen individuals of Paramecium aurelia. The animals are listed in the order in which they were washed, and no cases are omitted. Animals 12 and 14 lived three days after washing

(1) NO.	(2) 7TH WASH	(3) 8TH WASH	(4) CONDITION OF ANIMAL 24 HOURS AFTER WASHING	(5) CONDITION OF ANIMAL 48 HOURS AFTER WASHING	(6) CONDITION OF HAY WATER 48 HOURS AFTER WASHING	(7) CONTROL WASH WATER
1	Sterile	Sterile	Dead	Dead	Sterile	Sterile
2	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
3	Sterile	Contaminated	Dead	Dead	Sterile	Sterile
4	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
5	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
6	Sterile	Sterile	Dead	Dead	Sterile	Sterile
7	Sterile	Sterile	Dead	Dead	Sterile	Sterile
8	Contaminated	Contaminated	Dead	Dead	Contaminated	Sterile
9	Sterile	Sterile	Dead	Dead	Contaminated	Sterile
10	Sterile	Sterile	Dead	Dead	Sterile	Sterile
11	Sterile	Sterile	Dead	Dead	Sterile	Sterile
12	Sterile	Sterile	One alive	One alive	Sterile	Sterile
13	Sterile	Sterile	One alive	One alive	Contaminated	Sterile
14	Sterile	Sterile	One alive	One alive	Sterile	Sterile

datum and those with P. aurelia, it may be said that out of sixteen cases of successful sterilizations (nos. 5, 6, 9, 10, 13, 14, 15, and 16 of table 1 and nos. 1, 3, 6, 7, 10, 11, 12, and 14 of table 2) only two animals (nos. 12 and 14 of table 2) were able to survive complete inanition more than twenty-four hours and that the maximum duration of life for these two was three days.

GROWTH OF PARAMECIA IN PURE CULTURES OF BACILLUS
PYOCYANEUS

Material

It was desirable to use in this study an organism that is known to form a soluble toxin, in addition to being pathogenic to many animals. It was necessary that this organism should be one that grows well in hay water and preferably one that is not dangerously pathogenic to man. Bacillus pyocyaneus meets these requirements, although there is some difference of opinion concerning the seriousness of infections by this bacillus in man. The use of this species was suggested by Dr. Moyer S. Fleischer, of the Saint Louis University Medical School, and the bacteria used throughout the study came from his stock culture of this organism. Examinations made from time to time have shown that the organism is Gram negative and motile. It produces in agar the typical pigment of B. pyocyaneus and conforms in its growth in milk and in the various sugar media to the descriptions given for this bacillus in Bergy's Manual.[2] The pathogenicity of the organism was determined, after the culture had been maintained under laboratory conditions on agar for sixteen months, by injecting 0.5 cc. of a heavy suspension of the living organisms in physiological salt solution into each of three guinea-pigs. It was found that either subcutaneous or intraperitoneal injections in every case killed the animal in less than twelve hours. In one case the organism was recovered in pure culture from the subcutaneous exudate, from the peritoneal cavity, and from the heart-blood. Death of guinea-pigs resulting from such injections as described above was interpreted by Kolle and Wasserman ('03) as being due to the action of endotoxin.

The hay infusions containing the pure cultures of Bacillus pyocyaneus were prepared by inoculating sterile 0.2 per cent hay water contained in 100-cc. Erlenmeyer flasks and incubated at 35°C. for two days. These flasks were only partially

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[2] Bergy, David H. Manual of Determinative Bacteriology, 1923, pp. 123, 124.

filled, in order to provide a greater amount of surface for aeration. The hay water was prepared by the method previously described. In each case a large quantity of the medium was prepared in one large container to insure uniformity of composition, and then placed in a number of cotton-plugged flasks to be sterilized in steam by the intermittent method. This arrangement provided in each case enough hay infusion of uniform composition for at least five weeks. The pH of all media made by this method ranged from 6.8 to 8.3. Very little difference was found between the pH of sterile hay water and two-day cultures of B. pyocyaneus made

TABLE 3

Hydrogen-ion concentration of sterile hay water and of cultures made from it of B. pyocyaneus and of chance-mixed bacteria from the air

DATES	AUGUST 15, 1925	AUGUST 10, 1926
Methods	Colorometric	Hydrogen electrode potentiometer
Sterile hay water	6.8	8.14
Two-day culture of B. pyocyaneus	7.0	8.23
Ten-day culture of B. pyocyaneus	7.0	
Twenty-four-hour chance-mixed bacterial culture in same hay infusion	6.8	8.0

with the same hay infusions. The data in table 3 give the results of pH determinations made at two different times. The infusions used in all experimental work carried on during the months of June, July, and August, 1925, were made from Woods Hole pond water. From September, 1925, to June, 1926, St. Louis tap-water was used. During June, July, and August, 1926, infusions were made from distilled water and from St. Louis tap-water.

Methods

In establishing the growth of a pure line of paramecia in pure cultures of B. pyocyaneus a single animal was first isolated and permitted to undergo one division. Of the daughter

cells, one was used as the progenitor of all lines grown throughout the course of the experiment in the cultures of B. pyocyaneus; the other was used as the progenitor of all lines grown as controls in the chance-mixed bacterial cultures. The progenitor of lines to be grown in the pure cultures of B. pyocyaneus was washed by the method already described and then introduced by means of a sterile pipette into 0.5 cc. of the pure two-day culture of B. pyocyaneus contained in a sterile culture slide in a Petri dish. The immediate offspring of this animal were used as the progenitors of as many lines as was necessary to continue in the pure cultures. In each line that was thus continued transfers were made daily to fresh two-day cultures of bacteria. Animals were transferred in every case with sterile pipettes. Each transfer was made under a binocular and in each operation the lid of the Petri dish was raised barely enough to admit the pipette. After these lines had grown for a few days in the pure cultures, it was necessary to determine whether the cultures were still pure or whether foreign bacteria had been introduced with the washed paramecium or by any means. To do this a drop of each culture from each line, taken in each case after paramecia had grown in it for twenty-four hours, was plated out in agar by the usual bacteriological method. Where the cultures showed contamination, that line was discontinued and replaced by another started from one of the lines showing no contamination. Throughout the course of each experiment a record was kept for each line of the number of divisions per day and the number of deaths.

Experiments and observations

Paramecia grow at a high division rate and for apparently an indefinite period of time in pure cultures of Bacillus pyocyaneus. This occurs even in toxic cultures of B. pyocyaneus, provided the races of paramecia are first made tolerant to the toxic agent in the cultures. This fact has some significance in the light of certain discussions during recent years as to whether paramecia can maintain themselves in pure

cultures of any species of bacterium. Hargitt and Fray ('17), working with eleven of the common species of bacteria which inhabit hay infusions, found that paramecia did not maintain as high a division rate in pure cultures as when the animals grew in bacterial mixtures. Phillips ('22) found the same to be true for the twelve additional species that she studied.

The growth of four lines of P. aurelia for one hundred days in hay-infusion cultures of B. pyocyaneus and in chance-mixed bacterial cultures, similarly made with hay infusions, is summarized in figure 1 and in table 4. Since the cultures of B. pyocyaneus used were found to be toxic to P. aurelia, it was necessary to habituate the races to this toxic action before beginning the experiment. The method of such habituation is described and discussed in a later section (compare p. 99). The mean division rate of the four lines in the cultures of B. pyocyaneus for the one hundred days was 1.52, while that for the lines in chance-mixed cultures was 1.22, thus showing a higher division rate in the cultures of B. pyocyaneus.

So far as possible, the four lines of P. aurelia grown in cultures of B. pyocyaneus and the four control lines in the mixed-bacterial cultures were kept under uniform conditions. All lines were pedigreed stock having descended from one animal at the beginning of the hundred-day period. Conjugation was eliminated by the use of the daily isolation method. The temperature was controlled to between 22° and 25°C. The light was variable, but affected all lines similarly. The hay infusions used had a pH (determined colorometrically) of from 8.2 to 8.5 when sterile. Cultures of B. pyocyaneus made with this hay water did not develop any appreciable change in acidity or alkalinity. The pH of the chance-mixed bacterial cultures made from the hay water ranged from 7.1 to 8.0.

The growth of a number of lines of P. caudatum for fifty-five days in B. pyocyaneus and in chance-mixed bacterial cultures is summarized in figure 2 and in table 4. Here also the division rate is higher in the cultures of B. pyocyaneus, the

Division
 rate

Time in 10-day periods

I

Division
 rate

Time in 5-day periods

2
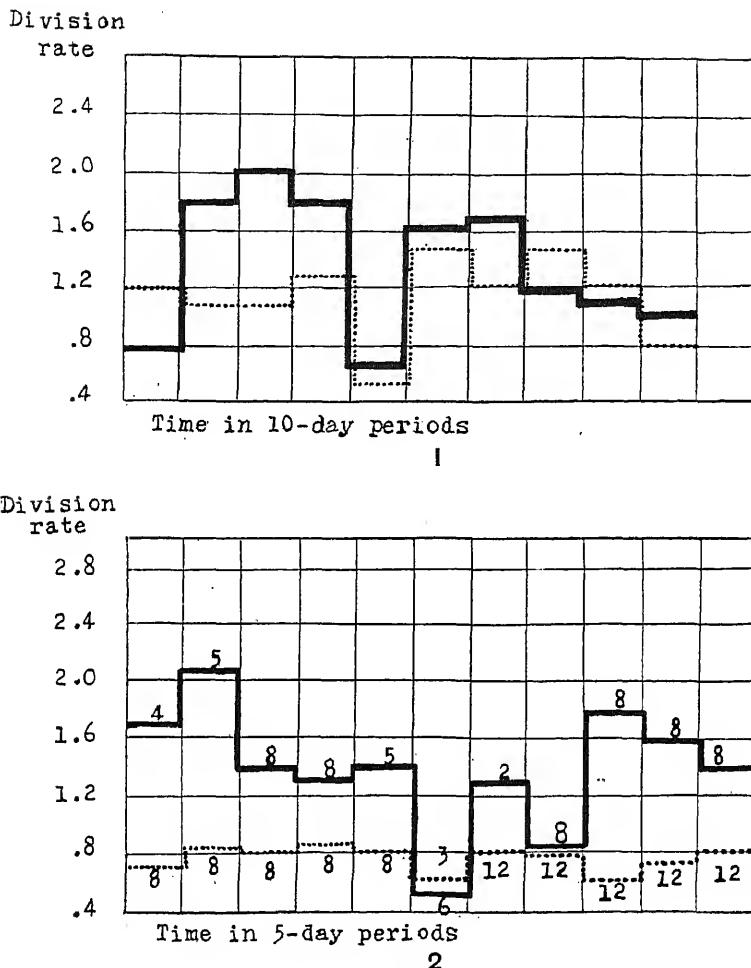
Fig. 1 Growth of four lines of P. aurelia for one hundred days in two-day cultures of B. pyocyaneus (heavy line) and in chance-mixed bacterial cultures (dotted line). February 26 to June 5, 1926. Each curve represents the average division rate of four lines again averaged for ten-day periods. Temperature, 22° to 25°C.

Fig. 2 Growth of a varying number of lines of P. caudatum for fifty-five days in two-day cultures of B. pyocyaneus (heavy line) and in chance-mixed bacterial cultures (dotted line). July 3 to August 26, 1925. Each curve represents the average division rate of a varying number of lines again averaged for five-day periods. The figures on the curves represent the number of lines of paramecia averaged into each five-day period. No control of temperature.

mean division rate being 1.4 as compared with 0.75 in the chance-mixed cultures. The same methods were used as for P. aurelia, except that there was no control of the temperature. The number of lines as shown in figure 2 varied from week to week. During most of the time eight lines were maintained, but during a few weeks the number was increased to twelve, and at the beginning of the experiment and later during periods of depression reduced to a smaller number. Each line was descended from one individual isolated at the

TABLE 4

Comparison of the mean division rates of three species of paramecia in hay-infusion cultures of B. pyocyaneus and of chance-mixed bacteria from the air

	MEDIUM	NUMBER OF DAYS	MEAN DIVISION RATE	PROBABLE ERROR	DIFFERENCE IN MEAN DIVISION RATES	PROBABLE ERROR OF DIFFERENCE	SIGNIFICANCE FACTOR
P. Aurelia	A. Mixed cultures	100	1.22	.50			
	B. Cultures of B. pyocyaneus	100	1.52	.53	.30	.73	.41
P. Caudatum	A. Mixed cultures	55	.75	.06			
	B. Cultures of B. pyocyaneus	55	1.4	.25	.65	.25	2.5
P. Calkinsi	A. Mixed cultures	85	1.48	.36			
	B. Cultures of B. pyocyaneus	85	2.54	.42	1.06	.55	1.9

beginning of the fifty-five-day period. These cultures of B. pyocyaneus were also toxic, and it was necessary, as with P. aurelia, to habituate all the lines of P. caudatum grown in them. The method of habituation is discussed later (compare p. 100).

The 0.2 per cent timothy-hay infusion used during the fifty-five-day period was made from Woods Hole pond water, and the animals used came from one individual isolated from a Woods Hole pond. The pH of the sterile timothy-hay infusion and of the B. pyocyaneus and mixed cultures made from it is shown in table 3 under the date of August 15, 1925.

A race of P. calkinsi grown in the cultures for a period of eighty-five days gave results similar to those for P. aurelia and P. caudatum. The results for this race are shown in figure 3 and in table 4. The mean division rate in the cultures of B. pyocyaneus was 2.54, while that in the chance-mixed cultures was 1.48.
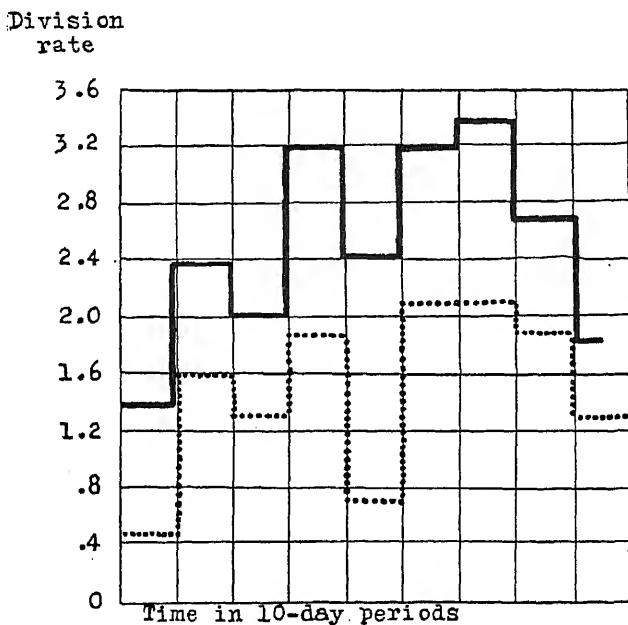
Division
  rate

Fig. 3 Growth of four lines of P. calkinsi for eighty-five days in two-day cultures of B. pyocyaneus (heavy line) and in chance-mixed bacterial cultures (dotted line). May 29 to August 22, 1926. Each curve represents the average division rate of four lines again averaged for ten-day periods. No control of temperature.

The 0.2 per cent hay infusions used for P. calkinsi during the eighty-five-day period were made from distilled water and contained 0.04 per cent NaCl. Woodruff ('21) and others have found the presence of salt to be necessary for the growth of this species. The temperature during the period was controlled to between 24° and 28°C. The pH of the infusion used is recorded in table 3 under the date of August 10, 1926.

The cultures of B. pyocyaneus made from the distilled-water hay infusions were not toxic for P. calkinsi. The possible significance of this is discussed in a later part of this report.

With the three races of paramecia used it is evident that the division rate in the cultures of B. pyocyaneus is higher than in the mixed cultures. When, however, the probable error for each mean division rate is taken into consideration, the significance factors, as shown in table 4, do not indicate that the higher mean division rates in the cultures of B. pyocyaneus are of as much significance as one might at first think. The method of determining this significance factor is that used by Phillips ('22). It is based upon the mathematical assumption that the numerical value representing the difference in the means under two experimental conditions is of significance if it contains the probable error of the difference more than three times. The significance factors shown in table 4 indicate that with no one of the three races of paramecia was the higher division rate in the cultures of B. pyocyaneus of such significance. The fact remains, however, that the average division rate in the cultures of B. pyocyaneus was at least as high as in the chance-mixed bacterial cultures, and this fact is significant in connection with the question whether pure cultures of bacteria can support continued growth of paramecia as well as chance-mixed cultures.

It may be possible, however, that the periods of high division rate of paramecia in the cultures of B. pyocyaneus is due to the stimulation by a toxic substance rather than to the superior nutritive qualities of these cultures for paramecia. Evidence is presented in another part of this report to show that an agent, which is toxic to paramecia, is present in hay-water cultures of B. pyocyaneus. It is a well-known fact that many poisonous substances may either retard or accelerate the general activity of protoplasm, depending upon the amount of the agent present (Pfeffer, '03, p. 264).

DEVELOPMENT BY PARAMECIA OF TOLERANCE TO THE TOXIC EFFECTS OF CULTURES OF BACILLUS PYOCYANEUS

Cultures of B. pyocyaneus made from 0.2 per cent hay water, prepared by diluting standard 1 per cent hay infusion with tap-water, were found to be toxic for P. aurelia, P. caudatum, and P. calkinsi; but under certain conditions races of these animals acquired a tolerance to this toxic action. This may be illustrated by the results obtained with P. aurelia (fig. 4). In this experiment a race, consisting of four lines descending from one animal isolated from Woodruff's stock culture of P. aurelia, was made tolerant to the toxic effects of two-day cultures of B. pyocyaneus by growing lines in the toxic cultures for a period of four days and then returning them for a period of recovery to chance-mixed bacterial cultures. After this four-day recovery period, the lines were again returned to the toxic cultures in which they were then able to grow during the remainder of the one hundred days of the experiment. Where no recovery period was provided, the lines introduced into the toxic cultures invariably died out. The heavy unbroken marking in figure 4 shows the average daily division rate during the hundred-day period of the lines in which tolerance had been acquired. Four control lines, also descended from the one individual isolated at the beginning of the experiment, were grown throughout the experiment in chance-mixed bacterial cultures. The average division rate of these controls is shown by the dotted marking. The toxic effect of B. pyocyaneus cultures was tested from time to time by introducing four lines of the animals taken from the controls into the toxic cultures. The fate of all of these test lines in which tolerance had not been acquired is shown in the figure. Each marking of dashes represents the average division rate of four of these test lines. In all, forty test lines were used, and no one of them was able to resist the toxic action, although the four lines in which tolerance had been acquired maintained a high division rate in the cultures of B. pyocyaneus throughout the entire period. Care was taken that the cultures of B. pyo-

cyaneus used for each test line were removed from the same flask as used that day for the four tolerant lines. In other words, there was no controllable difference at any time between the environment of the lines in which tolerance had been acquired and that of the non-tolerant test lines. The medium used during the hundred-day period was 0.2 per cent timothy-hay infusion made from St. Louis tap-water. More recent experiments, discussed in a later part of this report, show that distilled water cannot be used in making a hay-water culture of B. pyocyaneus that will be toxic to P. aurelia. The temperature of the experiment was controlled to within two degrees of 20°C.

It is clear that cultures of B. pyocyaneus were similarly toxic to P. caudatum and that tolerance was also acquired, but no attempt was made to collect as much evidence to show this as was collected for P. aurelia. In fact, P. caudatum was the species first used in the experiments, and with it the first indications of toxic action were observed. This was in May, 1925, when repeated attempts were made, without success, to grow lines of P. caudatum in two-day cultures of B. pyocyaneus. In June, 1925, an attempt was made for the first time to render a line of P. caudatum tolerant to the toxic action of cultures of B. pyocyaneus by providing periods

Fig. 4 Development of tolerance by P. aurelia to the toxic effects of two-day cultures of B. pyocyaneus. The heavy marking represents the average division rate in two-day cultures of B. pyocyaneus of four lines of P. aurelia which had acquired tolerance for the toxic effects of these cultures. Tolerance was acquired during the second five-day period, at which time the lines were grown for recovery in chance-mixed bacterial cultures. These lines were averaged for five-day periods.

The dotted marking represents the division rate of four control lines which were grown in chance-mixed bacterial cultures. These were sister lines to those grown in the cultures of B. pyocyaneus. The marking of dashes represents the fate of non-tolerant test lines of P. aurelia which were taken from the control lines and introduced into two-day cultures of B. pyocyaneus. Each line of dashes represents the average division rate of four of these non-tolerant lines on those particular days.

All animals used were descendants of one individual isolated at the beginning of the experiment. The temperature was controlled constantly to within two degrees of 20°C.
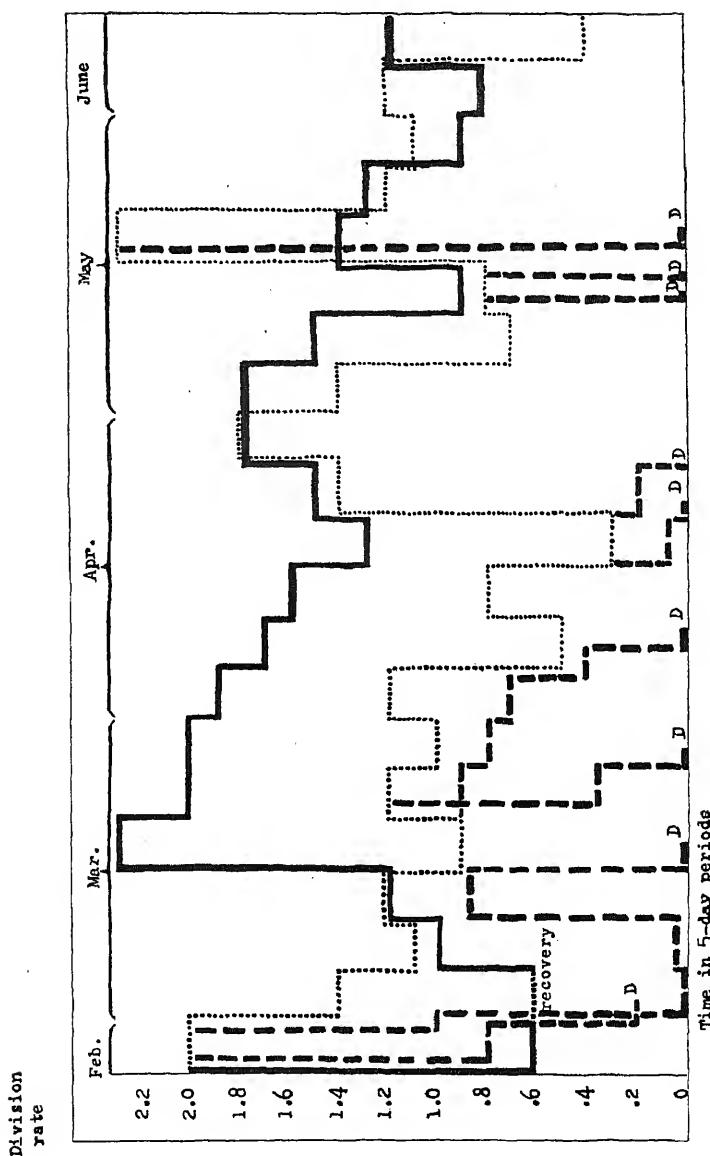
Figure 4

of recovery in chance-mixed bacterial cultures. Success came so quickly that it was not recognized as such. The lines treated began to divide rapidly in the toxic cultures and continued to do so for a period of fifty-five days, at the end of which time it was necessary to discontinue the study. The ease with which this tolerance was acquired weakened belief in the toxic action of the pyocyaneus cultures upon paramecium, and in a preliminary report of the investigation made (Philpott, '25) I stated that only the ten-day, and not the two-day cultures, were toxic. Later studies with P. aurelia and P. calkinsi as well as further studies with P. caudatum confirm the first conclusion, that even two-day cultures of B. pyocyaneus are toxic and that races of paramecia may acquire tolerance to the toxic agent.

Some data obtained with P. caudatum, which indicate the toxic action of the cultures of B. pyocyaneus and the ability of this species to acquire tolerance are represented in figure 5. The heavy marking (5, A) represents the division rate of eight lines of P. caudatum in ten-day cultures of B. pyocyancus. Previous to this time, these lines had grown for thirty days in chance-mixed bacterial cultures. These eight lines, as shown in the figure, grew well in the cultures of B. pyocyaneus for a period of ten days and then died out, with the exception of one line. This one line was saved by growing it for a five-day period in mixed bacterial cultures. It is the belief that this five-day recovery period brought about the development of tolerance in the animals. At the end of this recovery period an animal was returned to the ten-day cultures of B. pyocyaneus, and from its immediate progeny eight lines were produced which, as shown in the diagram, continued at a high division rate in the toxic cultures. The lines averaged in 5, B were sister lines to those shown in 5, A. These were tolerant lines and had previously grown for thirty days in cultures of B. pyocyaneus. These eleven tolerant lines, increased to twelve during the second five-day period and later to sixteen, did not show any depression in the division rate during the third five-day period when fatalities occurred in the non-tolerant lines shown in A.

All controllable factors in the lines that had been made tolerant and those not so treated were the same throughout the period of the experiment. The work was performed at Woods Hole during the summer of 1925. Hay water of 0.2 per cent, made from Woods Hole pond water, was used. More recent experiments, discussed in a later part of this
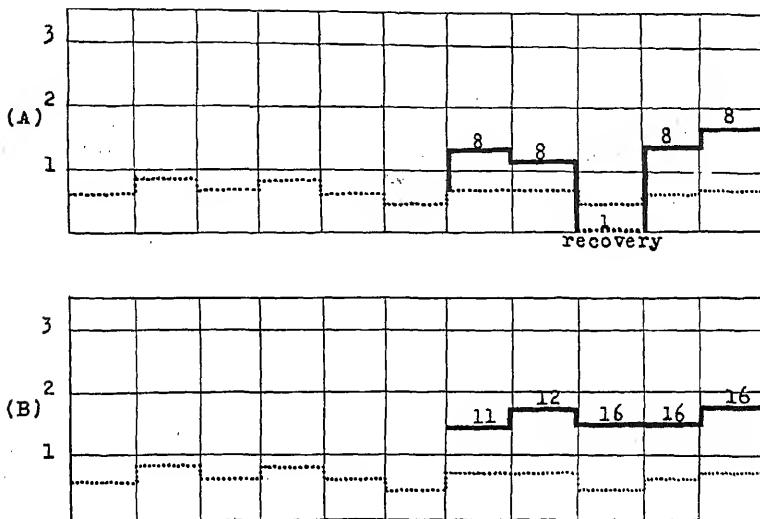
Fig. 5 (A) The heavy marking represents the division rate of eight lines of P. caudatum in ten-day cultures of B. pyocyaneus. These lines had previously grown in mixed-bacterial cultures. The dotted line represents the division rate of the controls in chance-mixed bacterial cultures. (B) The heavy marking represents the division rate of eleven to sixteen lines of P. caudatum in ten-day cultures of B. pyocyaneus. These were sister lines to those represented in (A), but had previously grown for thirty days in cultures of B. pyocyaneus. All division rates are averaged for five-day periods.

report, show that distilled water cannot be used in making a hay-infusion culture of B. pyocyaneus which is toxic to P. caudatum. All media used in this experiment were made up at the beginning of the fifty-five-day period in one large container and distributed in 100 cc. cotton-plugged flasks. Hence there could have been no variation in media from day to day. Each day the culture of B. pyocyaneus used for the

two sets of lines came from the same flask. There was no temperature control, but temperature variations were the same for all lines.

Under certain conditions, results have been obtained with P. calkinsi similar to those for P. aurelia and P. caudatum. Two-day cultures of B. pyocyaneus made from St. Louis tapwater were toxic to these animals, but they acquired tolerance

TABLE 5

Development of tolerance in four lines of P. calkinsi to growth in two-day cultures of B. pyocyaneus. The figures for each day indicate the number of divisions in the different lines for that day. The four lines in (A) were grown in two-day cultures of B. pyocyaneus except on certain days, as indicated by parentheses, when the animals were grown in chance-mixed bacterial cultures. All lines in (B) were grown daily in two-day cultures of B. pyocyaneus and there were no recovery periods in mixed cultures. When a death occurred in any line, a reserve animal in that line, from the preceding day when available, was used to take its place

		MAY						JUNE						
		29	30	31	1	2	3	4	5	6	7	8	9	10	11
(A)		1	(1)	(2)	1	1	2	0	0	2	2	1	2	3	3
		1	(0)	(2)	(2)	2	1	(1)	1	2	1	3	2	3	3
		1	(1)	(2)	(1)	2	1	(0)	1	2	1	3	3	2	2
		1	(0)	(3)	(1)	2	(1)	1	1	3	1	3	2	2	2
(B)		1	1	3	2	2	D								
		1	D	2	2	2	2	0	D						
		1	D	2	2	2	2	0	D						
		1	2	1	2	3	2	0	D						

to the toxic agent as shown in table 5. The eight lines shown here were descendants of one animal. Four lines of animals (table 5, A) were made tolerant by growing in two-day cultures of B. pyocyaneus and providing periods of recovery in mixed bacterial cultures. The recovery periods are indicated by figures in parentheses. The four sister lines (table 5, B) were grown under the same conditions as for the four tolerant lines (table 5, A), except that no recovery periods were provided. These lines (5, B) were not able

to develop tolerance to the toxic agent in the culture, and all were dead at the end of the eighth day. As in previous experiments, the cultures of B. pyocyaneus used for all lines during any one day came from one flask. The hay infusion used was made from St. Louis tap-water to which 0.2 per cent NaCl was added. The animals used were from Woodruff's stock pedigreed culture of P. calkinsi.

It was found, however, that when distilled water was used in making the hay infusion, the cultures of B. pyocyaneus made from it were not toxic to P. calkinsi. During the summer of 1926, repeated tests for toxic effects were made by introducing lines of P. calkinsi into such cultures of B. pyocyaneus, and in no case was there an evidence of toxic action. Care was taken to prepare these cultures in the same manner as all toxic cultures had previously been made, except that distilled water was used instead of tap-water. When, finally, it was evident that the distilled-water cultures were non-toxic to the lines of P. calkinsi, the same lines of animals were introduced into cultures of B. pyocyaneus made from St. Louis tap-water, and the usual toxic effects were obtained. In the cultures of B. pyocyaneus made from the St. Louis tap-water all of the lines died out within a week, in spite of the fact that they had previously grown for a long period of time in the non-toxic cultures of B. pyocyaneus made with distilled water. The hydrogen-ion concentration of these cultures as determined by means of the hydrogen-electrode potentiometer were 8.2 for the toxic and 7.67 at one time and 8.2 at another for the non-toxic. Since, by this method of determining hydrogen-ion concentration, all CO_2 is driven out of solution, it is probable that these cultures were less alkaline than the above readings indicate. The same lines of animals and the same strain of B. pyocyaneus were used in these toxic and non-toxic cultures, so that it is improbable that the difference was due to variations in these organisms.

Later, results similar to those described for P. calkinsi were obtained for P. caudatum and P. aurelia. Two experiments were carried out with each of these species where, in

each case, four lines were grown in cultures of B. pyocyaneus
made from distilled water, four in cultures of B. pyocyaneus
made from tap-water, and four in chance-mixed bacterial
cultures. In each experiment the four lines in the cultures
of B. pyocyaneus made from tap-water died within from one
to seven days, while all other lines continued to live (tables
10 and 11). No reason for the non-toxic action of the cultures
in distilled-water media is apparent unless it is due to the
absence of certain buffer substances in the distilled water that
were present in tap-water.

DURATION OF THIS ACQUIRED TOLERANCE

The tolerance acquired by a race of paramecia to the toxic
action of a culture of B. pyocyaneus is not permanent. On
March 22, 1926, four tolerant lines of P. aurelia, which had
previously grown for fifteen days in toxic cultures of B.
pyocyaneus, were introduced into chance-mixed bacterial cul-
tures and grown there for twelve days or for what amounted
to an average of fourteen generations in the four lines. The
four lines were then returned to the toxic cultures of B.
pyocyaneus in which they died within three days. Evidently,
the tolerance which the lines had previously acquired was
lost during the period of growth in the mixed bacterial cul-
tures. During all of this time four sister tolerant lines con-
tinued to grow in the toxic infusions at a high division rate
with no deaths. These results are shown in figure 6. The
four tolerant lines referred to here are the same as have
been described in connection with figure 4 of this paper.

A repetition of this experiment is also shown in figure 6.
On May 2nd, from the same four tolerant lines of P. aurelia,
four lines were introduced into chance-mixed bacterial cul-

Fig. 6 Results of an experiment to determine the duration in races of P.
aurelia of the acquired tolerance to the toxic action of cultures of B. pyocyaneus.
The heavy marking represents the average division rate of four tolerant lines of
P. aurelia growing in two-day cultures of B. pyocyaneus from February 26 to
June 5, 1926. The division rates have been averaged for five-day periods. Each
of the dotted markings represents the fate of four of the tolerant lines which
were grown in chance-mixed bacterial cultures for a period of time and then
returned to the toxic cultures of B. pyocyaneus.
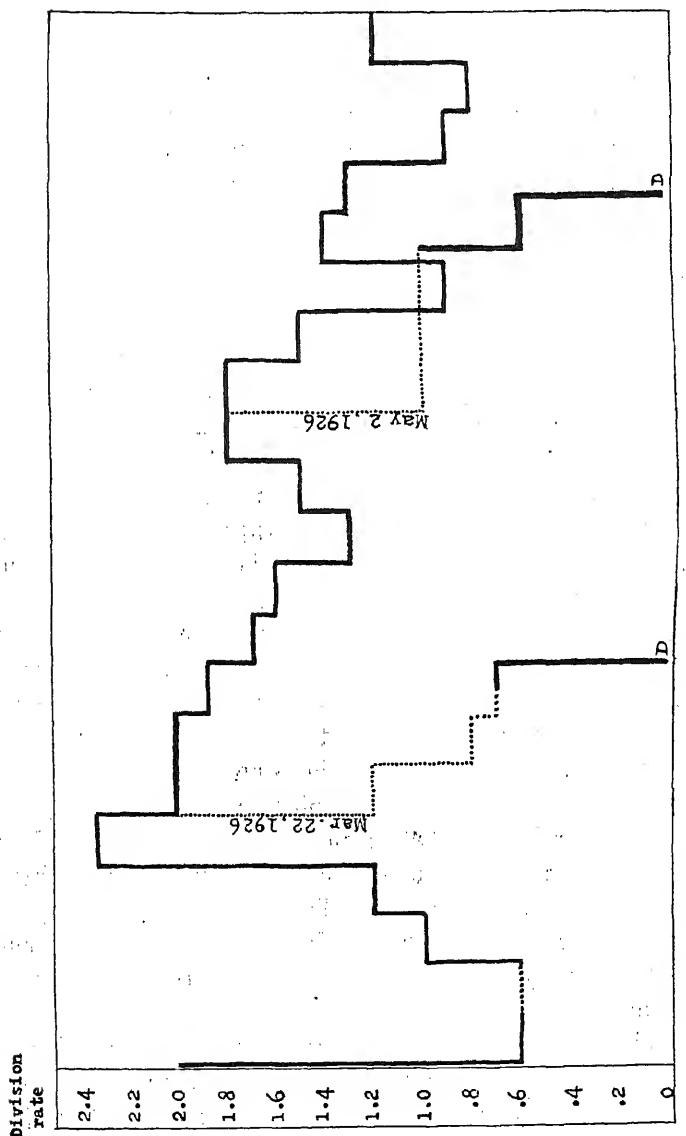
Figure 6

tures and grown there for seventeen days. During this period an average of ten generations occurred in the four lines. These four lines were then returned to cultures of B. pyocyaneus, in which they died within six days. During the entire time of the experiment four sister tolerant lines continued in the toxic cultures with no fatalities. It is here evident again that the acquired tolerance was lost, this time during a period of seventeen days' growth, or ten generations, in mixed bacterial cultures.

NATURE OF THE TOXIC AGENT IN CULTURES OF BACILLUS PYOCYANEUS

There are several substances known to be present in cultures of Bacillus pyocyaneus, any one of which might produce a toxic effect upon paramecium. Wasserman ('96) concluded from the result of his investigations that this organism produces both a soluble exotoxin and an endotoxin. Although the strength of the exotoxin never approaches that of diphtheria and tetanus, the blood of the animals immunized against it contains antitoxic substances. Acklin ('25) has shown that various strains of B. pyocyaneus growing in relatively simple, albuminoid-free media retain their virulence and ability to produce toxin. Wasserman ('96) reported that animals actively immunized with living culture masses of B. pyocyaneus, that is with the endotoxin, develop only bacteriolytic antibodies in their blood serum.

Emmerich and Low ('99) report that filtrates of old broth cultures of B. pyocyaneus contain a ferment-like substance which they call 'pyocyanase.' This is able to destroy other bacteria, apparently by lysis. They describe pyocyanase as being exceedingly thermostabile, since it resists boiling for several hours.

Bulloch and Hunter ('00) report the presence in cultures of B. pyocyaneus of another thermostabile substance which they called 'pyocyanolysin.' This agent is capable of producing a hemolyzing effect. Jordan ('03) thought, however, that this hemolyzing effect was due to the alkalinity of the filtrates.

Petty ('21) has shown that B. pyocyaneus growing in egg-broth gelatin and in a synthetic broth produces measurable quantities of hydrocyanic acid. He found that the amount varied with different strains of B. pyocyaneus and with the media and the pH.

The attempt has been made in the present study to use the method of elimination as the means of determining which, if any, of the above agents may be responsible for the toxic action of cultures of B. pyocyaneus upon paramecia. It was recognized at the outset that the reliability of this method depends upon the extent to which all possible causative agents are taken into consideration. There may, of course, be other toxic agents in cultures of pyocyaneus in addition to those above cited; but, in view of the many studies which have been made relative to the toxic action of this organism, the existence of such agents seems improbable.

The results obtained indicate that it is something dissolved in the cultures of B. pyocyaneus, and not the endotoxin which serves as the toxic agent. Attention has already been called to the fact that cultures of this organism in hay infusion made from distilled water were not toxic to any of the three species of paramecia used in this study. Neither did races of paramecia grown in non-toxic cultures of B. pyocyaneus develop any resistance that enabled them to grow in toxic cultures of these bacilli. Since paramecia growing in these non-toxic cultures are feeding upon the living bacilli, it would seem that the endotoxin is not the toxic agent. Otherwise one must suppose that the endotoxin is non-toxic under one set of conditions and toxic under the other. The conclusion that the endotoxin is not responsible for the toxic action is further strengthened by the fact that filtrates of cultures of B. pyocyaneus have been found to contain a soluble toxic agent.

That an agent toxic to paramecium is produced in the medium in which pyocyaneus bacilli grow is indicated by the results of experiments which show that tolerance in paramecia may be developed by subjecting the animals to the

effects of filtrates of cultures of B. pyocyaneus. Table 6 shows the results of one of these experiments. For use in the experiments filtrates of cultures of B. pyocyaneus were made by passing two-day cultures of this organism through Berkefeld filters. Four lines of P. aurelia were grown in these filtrates as shown in table 6. On August 14th, the animals were grown in the filtrate for a twenty-four-hour

TABLE 6

Development of tolerance by P. aurelia to growth in two-day cultures of B. pyocyaneus. Numbers in parentheses are the divisions for the day in filtrates of cultures of B. pyocyaneus. The italic numbers represent divisions per day in two-day cultures of B. pyocyaneus. Numbers without parentheses or italics represent divisions in chance-mixed bacterial cultures. The four lines (A) were rendered tolerant by growing during four different days in filtrates of B. pyocyaneus. Four sister control lines (B) were grown during the time in chance-mixed bacterial cultures and were not subjected to the filtrate. These four lines, when introduced into cultures of B. pyocyaneus, could not resist its toxic action and died as shown on the days marked 'D' in the table

		14	15	16	17	18	19	Transferred to two-day cultures of B. pyocyaneus	20	21	22	23	24	25	26	27	28	29	30	Continued in mass cultures
													AUGUST							
(A)		(0)	1	1	(0)	(2)	(1)		*1*	*2*	*2*	*2*	*2*	*2*	*3*	*1*	*2*	*2*	*3*	
		(0)	1	1	(0)	(D)	(1)		*1*	*2*	*2*	*2*	*3*	*2*	*3*	*0*	*2*	*3*	*3*	
		(0)	1	2	(0)	(0)	(1)		*1*	*1*	*2*	*2*	*0*	*2*	*3*	*2*	*2*	*2*	*2*	
		(0)	1	1	(1)	(1)	(1)		*2*	*2*	*3*	*3*	*3*	*2*	*3*	*2*	*2*	*2*	*3*	
(B)		1	0	2	1	0	1		*1*	*1*	*2*	*0*	*0*	*1*	*0*	*0*	*0*	D		
		1	0	D	2	0	1		*2*	*0*	*0*	*0*	*1*	*0*	*0*	*0*	D			
		1	0	1	1	0	2		*2*	*2*	*1*	*1*	*1*	*1*	*0*	*1*	*1*	*0*	D	
		1	0	2	1	0	1		*2*	*0*	*1*	*0*	*1*	*1*	*0*	*0*	*0*	*0*	D	

period. This was accomplished by placing the sterile filtrate in the cavities of depression slides contained in Petri dishes and introducing with each animal barely enough miscellaneous bacteria to supply food. The four lines were then allowed to recover for a two-day period in chance-mixed bacterial cultures, after which they were again grown in filtrates of B. pyocyaneus for three days. At the end of this period, the four lines were introduced into two-day cultures of B. pyocyaneus (table 6), where they were able to maintain a high division rate. The four control sister lines, which were not

grown during the first six days in the toxic filtrates, but in non-toxic mixed bacterial cultures, were unable, when introduced into cultures of B. pyocyaneus, to resist the toxic effect. It thus appears that the four lines subjected at intervals during a six-day period to filtrates of B. pyocyaneus acquired a tolerance to the toxic agent. Unfortunately, it was impossible to continue the four tolerant lines by the daily isolation method after August 30th. Mass cultures were made, however, and these continued for three weeks. All the lines of paramecia used in the experiment, including the controls, descended from one animal isolated at the beginning. After the sixth day, all tolerant and control lines were grown each day in portions of a culture of B. pyocyaneus removed from one flask. This served to keep the environment the same for all lines. The temperature was controlled to between 24° and 28°C.

A similar experiment was performed with P. caudatum, the results of which are shown in table 7. Here the results are not so satisfactory. The four lines that were grown at intervals in filtrates of B. pyocyaneus and then introduced into cultures of this organism were, as expected, tolerant to the toxic action. Two of the control lines which had been subjected to the filtrates died in accordance with expectations shortly after being introduced into the toxic cultures. The other two control lines survived. The fact that these two surviving control lines went through a critical period during the first six days of their growth in the toxic cultures may be significant. During the third, fourth, and fifth days there were no divisions in either of these lines. During the sixth day in the cultures of B. pyocyaneus there was only one division in each of these lines. During the same period of time the four sister tolerant lines, as shown in table 7 (A), were growing in the same cultures of B. pyocyaneus and at a high division rate. A depression period seems, therefore, to have occurred in these control lines when they were introduced into the toxic cultures. After the first six days in the toxic cultures, the two surviving control lines increased their division rates. My interpretation of these data is that the two control

lines of P. caudatum were able to survive and recover from the toxic effects of the cultures of B. pyocyaneus and that they acquired a tolerance for the toxic substance in the culture as a result of their experience. The method used in rendering lines of P. caudatum tolerant by means of toxic filtrates were the same in every way as those used with P. aurelia and described above.

TABLE 7

Development of tolerance by P. caudatum to growth in two-day cultures of B. pyocyaneus. Numbers in parentheses are the divisions for the day in filtrates of cultures of B. pyocyaneus. The italic numbers represent divisions per day in two-day cultures of B. pyocyaneus. Numbers without parentheses or italics represent divisions in chance-mixed bacterial cultures. The four lines (A) were rendered tolerant by growing during three different days in filtrates of B. pyocyaneus. Four sister control lines (B) were grown during the time in chance-mixed bacterial cultures and were not subjected to the toxic filtrates. Two of these lines when introduced into the cultures of B. pyocyaneus could not resist its toxic action and died as shown on the days marked 'D' in the table. The other two lines continued to grow after a period of depression

									AUGUST										
	14	15	16	17	18	19	20	21	Transferred to two-day cultures of B. pyocyaneus	22	23	24	25	26	27	28	29	30	Continued in mass cultures
(A)	2	0	2	(0)	1	(1)	(0)	0		*3*	*3*	*3*	*3*	*4*	*4*	*3*	*4*	*3*	
	0	D	2	(1)	0	(1)	(1)	0		*3*	*3*	*2*	*3*	*4*	*3*	*4*	*4*	*3*	
	0	1	1	(0)	0	(0)	(0)	1		*2*	*3*	*3*	*3*	*3*	*3*	*3*	*4*	*4*	
	3	0	2	(0)	0	(1)	(0)	1		*3*	*3*	*4*	*3*	*4*	*4*	*4*	*4*	*4*	
(B)	2	0	2	1	1	3	4	0		*3*	0	*1*	D						
	0	D	2	1	1	2	4	0		*2*	0	D							
	0	1	1	2	1	2	2	0		*1*	*2*	0	0	0	1	*3*	*4*	*3*	
	3	0	2	1	1	3	3	0		*2*	*2*	0	0	0	1	*3*	*4*	*8*	

It has been impossible to demonstrate directly that the filtrates of B. pyocyaneus are toxic for paramecia. The filtrate when first obtained is, of necessity, sterile. When paramecia are introduced into this sterile filtrate, they die of starvation, unless miscellaneous bacteria are added as food. Thus it is impossible to determine directly whether the death of an animal that has been introduced into the supposedly toxic filtrate is a result of toxic action or of starvation.

It appears from another experiment that the toxic agent in cultures of B. pyocyaneus is destroyed by heat (table 8). In this experiment eight lines of P. aurelia were grown from

TABLE 8

Results of an experiment to determine whether cultures of B. pyocyaneus that had been heated had lost their ability to induce the development of tolerance in lines of P. aurelia. May 2 to May 19, 1926. Each figure represents the number of divisions for the day

Growth for fourteen days in cultures of Bacillus pyocyaneus which had been heated to 80°C. for fifteen minutes

(A)														Transferred to two-day cultures of B. pyocyaneus			
2	1	1	2	2	1	1	2	2	1	1	1	2	1		2	0	D
2	1	2	1	3	2	2	2	2	0	1	1	1	1		3		D
2	1	2	1	3	2	2	0	2	2	2	1	1	1		2	0	D
0	1	2	1	2	1	1	1	1	1	2	2	1	1		2		D

Growth for fourteen days in cultures of B. pyocyaneus which had been heated to 100°C. for fifteen minutes

(B)														Transferred to two-day cultures of B. pyocyaneus					
2	1	1	2	1	1	2	2	2	1	2	1	1	1		3	0	0	D	
2	1	2	1	2	1	2	2	2	1	1	1	2	1		0	0	D		
2	1	2	1	1	1	1	2	2	2	1	1	1	1		0	0	1	0	D
0	1	1	1	1	1	2	2	2	2	0	2	2	1		2	0	0	0	D

Growth for fourteen days in chance-mixed bacterial cultures

(C)														Transferred to two-day cultures of B. pyocyaneus				
2	2	1	1	1	2	0	0	D	2	1	0	0	2		2	D		
2	3	2	1	1	2	0	0	0	1	2	0	1	1		2	D		
2	2	2	1	2	0	1	0	2	0	0	0	1	1		2	D		
2	2	2	1	1	2	1	D	D	2	2	1	1	1		2	0	0	D

Growth of control tolerant sister lines in two-day cultures of B. pyocyaneus

(D)														Continued in two-day cultures of B. pyocyaneus								
2	1	2	2	2	2	2	2	2	0	2	1	1	1		3	2	1	2	2	2	1	1
2	1	2	2	2	1	2	2	1	2	1	1	1	1		2	2	1	2	2	2	1	2
2	2	1	2	1	0	2	2	2	0	1	1	0	1		0	1	0	1	1	1	0	1
3	2	2	1	1	1	1	2	0	1	1	1	1	1		1	2	2	2	1	1	1	2

day to day in cultures of B. pyocyaneus that had been heated to a temperature high enough to kill all bacilli and to destroy anything in the nature of a toxin. The temperatures used were 80° and 100°C. for fifteen minutes. These lines of paramecia were carried by means of the daily isolation

method for a period of fourteen days in the boiled cultures of B. pyocyaneus. In transferring each animal into the culture that had been heated, enough miscellaneous bacteria were carried along with the paramecium to provide food for the following day, but care was taken to keep this bacterial contamination at a minimum. The results given in table 8 show no evidence that the heated cultures were toxic to paramecia. The lines growing in these heated cultures (A and B) displayed division rates as high as those for the control lines (C) in the chance-mixed bacterial cultures. At the end of the fourteen-day period the eight lines of paramecia were introduced into two-day cultures of B. pyocyaneus. Each of these lines died in a few days after introduction into the toxic cultures, which shows that there was nothing in the heated culture that had the effect of stimulating the development of tolerance in the race of paramecia. During the time that these lines were dying in the toxic cultures of B. pyocyaneus, four control tolerant sister lines (D) grew day by day in the same toxic cultures used for the dying lines and with no depression in division rate. This indicates that a toxic agent was responsible for the death in all lines indicated in A, B, and C. The control tolerant lines shown in D of this figure are the same as those described in figure 4 of this paper.

The fact that a boiling temperature for fifteen minutes destroys the effectiveness of the toxic action in a culture of B. pyocyaneus eliminates the possibility of this action being due to the presence of either pyocyanase or pyocyanolysin, because these substances are able to resist boiling for several hours (compare p. 108).

The experiment showing the effect of heat upon the toxic agent does not, however, eliminate hydrocyanic acid as the responsible factor, since boiling for fifteen minutes might drive off this volatile substance. There are, however, indications that HCN is not responsible for the toxic action of B. pyocyaneus upon paramecia. In the first place, it is probable that very little of the HCN, known to be produced in the cultures of B. pyocyaneus used in these experiments, could have

existed in the free state, since these cultures had a reaction on the alkaline side of neutrality. In such a medium as alkaline hay infusion it is probable that much of the free HCN was converted into salts which are not so toxic as free HCN.[3] In the second place, certain facts indicate the absence of any correlation between the degree of toxic action of certain cultures and the amount of HCN they were found to produce in a given time. Indeed, certain cultures of B. pyocyaneus found to be non-toxic to paramecia produced in a given time as much HCN as was produced in toxic cultures (table 9).
· The method of estimating the amount of HCN was that used by Petty ('21). In each determination 200 cc. of hay

TABLE 9

Estimated amount per cubic centimeter of HCN liberated in seven days in cultures of B. pyocyaneus made from distilled-water hay infusions and from tap-water hay infusions. Each culture was incubated for seven days at 35°C. The pH determinations were made by colorometric methods

	DISTILLED-WATER CULTURES	pH	TAP-WATER CULTURES	pH
First determination	.00033 mg.	7.0	.00018 mg.	8.0
Second determination	.00044 mg.	7.0	.00041 mg.	8.5

water in a 250-cc. flask was inoculated with Bacillus pyocyaneus and incubated for seven days at 35°C. During this period air, filtered through soda lime, was passed through the culture and into 4 per cent KOH. At the end of this seven-day period the KOH solution was added to the seven-day culture of B. pyocyaneus, the whole was acidified with sulphuric acid and distilled into 4 per cent KOH. To the distillate 1 cc. of yellow ammonium sulphide was added, the whole was evaporated to dryness on a water-bath and the residue was extracted with three 10-cc. portions of acetone. The acetone solution was then evaporated to dryness and water added. To this solution of potassium sulphocyanide fifteen drops of 5 per cent ferric chloride solution was added,

[3] Sollman, Torold. A Manual of Pharmacology, third edition, 1926, p. 774.

which converted the KCNS into blood-red ferric thiocyanate. The amount of the latter was determined colorometrically by comparing with freshly made KCNS solutions of known strength to which ferric chloride solution had been added.

The results shown in table 9 indicate that the amount of HCN present in the toxic two-day cultures of B. pyocyaneus used in this study was very small. Especially is this evident when one considers the fact that the amount shown in the table represents the total amount produced in a culture of B. pyocyaneus in seven days, and not the amount present at any one time; and that the estimated amount includes not only the free HCN present in the culture during the period, but also the HCN derived from the salts of cyanide that were formed in the culture. As has been pointed out, all of the hay-infusion cultures of B. pyocyaneus used in this study had a pH on the alkaline side of neutrality. It is probable that much of the HCN in each culture was converted into salts, as fast as it was formed, with the result that little of it existed at any one time in its free state.

Table 9 also indicates that a greater amount of HCN was present in the hay-infusion cultures of B. pyocyaneus that were made with distilled water than in those made with tap-water. Since the distilled-water cultures of B. pyocyaneus, in which the greater amounts of HCN existed, were non-toxic to paramecia, while similar cultures made from tap-water were toxic, it would seem that HCN is not the toxic agent. One may conclude that there is no correlation between the toxicity and the amount of HCN present under the two conditions, because the amount of HCN present in the non-toxic cultures is, if anything, slightly more than in the toxic cultures.

This non-toxic action of cultures of B. pyocyaneus made from distilled water was first observed in connection with the growth of P. calkinsi in cultures of B. pyocyaneus as has been previously explained (p. 105). To make sure of this difference in toxicity in the two types of cultures further experiments were undertaken with P. caudatum and P. aurelia. The

results of this study are shown in tables 10 and 11. The experiments were conducted at the same time that the HCN

TABLE 10

Difference of the effect upon Paramecium aurelia of growth in two-day cultures of B. pyocyaneus made from distilled water (A) and those made from tap-water (B). All lines were descendants of one individual isolated at the beginning of the experiment. The pH determination was made once only, but the same medium was used throughout the experiment. The pH determinations were made by colorometric methods

	JANUARY									
	20	21	22	23	24	25	26	27	28	
(A)	0	1	1	1	1	2	0	2	1	pH of culture 7.0
	0	2	1	1	1	2	1	1	1	
	1	0	1	1	1	2	1	1	2	
	1	1	0	1	1	1	1	2	1	
(B)	0	1	D							pH of culture 8.5
	0	0	1	0	1	D				
	0	1	1	1	D					
	0	0	0	1	D					

TABLE 11

Difference of the effect upon Paramecium caudatum of growth in two-day cultures of Bacillus pyocyaneus made from distilled water (A) and those made from tap-water (B). All lines were descendants of one individual isolated at the beginning of the experiment. The pH determination was made once only, but the same medium was used throughout the experiment. The pH determination was made by colorometric methods

	FEBRUARY									
	3	4	5	6	7	8	9	10	11	
(A)	1	2	2	1	1	1	2	1	1	pH of culture 7.0
	1	2	1	1	0	1	2	1	1	
	1	2	1	0	0	1	2	1	0	
	1	2	1	1	1	1	1	1	1	
(B)	1	0	0	0	D					pH of culture 8.5
	1	0	0	0	D					
	1	0	D							
	1	0	0	D						

determinations were being made. The distilled-water hay infusions used in making the cultures of B. pyocyaneus for the HCN determinations and the cultures of B. pyocyaneus in which the races of paramecia were grown were made up at one time in one large container. Likewise, the tap-water infusions used for both purposes were made in one container. Thus there could have been little variation of media in the similar cultures used for the different purposes.

It is evident from the results presented in tables 10 and 11 that the cultures of B. pyocyaneus which were made from tap-water hay infusions and which, as shown in table 9, contained the smaller amount of HCN, were toxic to P. aurelia and P. caudatum, while similar cultures which were made from distilled-water hay infusions and which contained a greater quantity of HCN were non-toxic.

The alkalinity or acidity of the hay-infusion cultures is not responsible for the toxic action upon paramecia. The hay-infusion cultures of B. pyocyaneus which were toxic to P. aurelia had a pH of from 8.2 to 8.5. The cultures that were toxic to P. caudatum had a pH of 7.0, while the non-toxic, mixed bacterial cultures, used as controls, had a pH ranging from 7.1 to 8.0. The cultures of B. pyocyaneus that were toxic to P. caudatum had a pH of 7.0, while the non-toxic, mixed bacterial cultures, used as controls, had a pH of 6.8. These determinations were made by colorometric methods.

It may also be mentioned that the agency in cultures of B. pyocyaneus that is toxic to paramecia does not cause immediate death of these animals. Death in any line usually results after a period of from five to ten days of growth in the toxic cultures, during which a high division rate of the paramecia is not uncommon. This may be compared with the 'period of incubation' of a toxin or time elapsing between inoculation and death as seen in higher animals. Thus a guinea-pig usually dies in or after the fourth day following inoculation with a lethal dose of diphtheria toxin, and such incubation periods are a distinguishing feature of toxins.

The facts above presented seem to eliminate the possibility that the toxic action of the cultures of B. pyocyaneus is due to endotoxin, to pyocyanase, to pyocyanolysin, to hydrocyanic acid, or to hydrogen-ion concentration. They point to the soluble toxin of B. pyocyaneus as being the toxic agent unless there is some other poisonous substance produced in the culture by the bacillus that is either thermolabile or capable of being volatilized and driven out by boiling temperatures.

In the absence of exact knowledge regarding the chemical nature of what are called 'toxins,' it is perhaps as justifiable to call the lethal agent a 'toxin' as a 'deleterious substance,' and to conclude that an 'immunity' to B. pyocyaneus can be developed in paramecium comparable with that produced in higher animals. Such was the terminology used in the preliminary reports of the present work (Philpott, '25, '26). The subsequent discovery of Petty's account ('21) of the production of hydrocyanic acid by Bacillus pyocyaneus has led to a more cautious statement, although it does not seem that hydrocyanic acid is the effective agent. It has seemed advisable, therefore, to use the term 'toxic substance' and 'tolerance' throughout, although it is probable that we are dealing with a true case of immunization against a toxin. Similar results with other toxins and with other protozoa are needed to establish the conclusion that races of protozoa may be immunized to toxins.

GROWTH OF PARAMECIA IN PURE CULTURES OF BACILLUS ENTERITIDIS

In contrast with the foregoing relationships in cultures of Bacillus pyocyaneus, the evidence thus far obtained indicates that paramecia will not grow in pure cultures of Bacillus enteritidis. All attempts that have been made to render pure lines of paramecia capable of growth in pure cultures of B. enteritidis have failed.

Bacillus enteritidis Gaertner was selected as an organism to be used in this study, because of its pathogenic effects. It is perhaps the commonest cause of food poisoning and affects

a wide variety of vertebrate animals. The poisonous product
of the growth of this organism is known to be an endotoxin,
and not a true toxin. No antitoxic substances are known to be
developed. The strain of the organism used was obtained
from the American Type Culture Collection of 637 S. Wood
Street, Chicago, Illinois.

TABLE 12

*Growth of twelve lines of Paramecium caudatum in pure cultures of Bacillus
enteritidis. The number of days indicated is the entire time the line grew
in cultures of B. enteritidis before dying out. The division rate shown for
the control lines in the mixed bacterial cultures is the average number of
divisions per day for the number of days during which the line in the cultures
of B. enteritidis continued to grow. In each case the animals in the control
belonged to the same pure line as those in the cultures of B. enteritidis*

NO.	DATE	NUMBER OF DAYS	AVERAGE DIVISION RATE PER DAY	AVERAGE DIVISION RATE IN CONTROL
	1925			
1	February 24	5	.4	.6
2	February 24	5	.4	.6
3	March 5	2	.6	1.5
4	March 5	3	1.0	1.0
5	March 4	3	1.0	1.5
6	March 4	4	1.0	1.5
7	March 5	2	.5	1.5
8	March 5	3	.3	1.0
9	March 12	2	.5	1.0
10	March 12	2	.5	1.0
11	March 13	4	.5	2.0
12	March 13	4	.5	2.0
	Mean	3.25	.6	1.26

The cultures of B. enteritidis were made by inoculating 0.2
per cent sterile hay water contained in cotton-plugged flasks
and incubating for two days. Quantitative bacteriological
analyses indicated that the bacilli grew profusely in the
medium.

The animals used belonged to a pure line of P. caudatum
descended from one individual isolated from Saint Louis
pond water on February 23, 1925.

The results of the growth of twelve typical cases of P. caudatum in enteritidis and in mixed cultures are shown in table 12. It appears that the cultures of B. enteritidis are toxic to P. caudatum. The lines of paramecia were not killed immediately when introduced into the cultures, but continued to live for an average of 3.25 days. During the period when the lines in the cultures of B. enteritidis were dying the sister control lines in the mixed cultures were growing normally, showing that death in the toxic cultures was due either to the condition of the medium or to the nature of the food. The average division rate for the twelve lines in the cultures of B. enteritidis was 0.6, as compared with an average of 1.26 for the control lines in the chance-mixed bacterial cultures.

Attempts were made without success to habituate lines of P. caudatum to growth in cultures of B. enteritidis. No tolerance was developed in the lines even when three recovery periods were provided. Neither was it evident that the lines developed any greater degree of sensitiveness to enteritidis bacilli as a result of the periods of growth and recovery.

GROWTH OF PARAMECIA IN MEDIA CONTAINING DIPHTHERIA TOXIN

In the preceding section attention has been called to the difficulty of determining whether the resistance acquired by races of paramecia to the toxic action of cultures of B. pyocyaneus represents an acquired tolerance to a poison or a true case of immunization to a toxin. It would be easier to study the phenomenon of immunization in races of paramecia, if such exists, by working with standard toxins. Minute and measured amounts of such material could be added to culture media of known composition, and if toxic action resulted there could be no question about the causative agent. At the present stage of this investigation, diphtheria toxin is the only one of this type that has been employed.

The diphtheria toxin used was supplied through the kindness of Dr. Mazyck P. Ravenel, of the Department of Medical Bacteriology and Preventive Medicine, and director of the

Public Health Laboratory of the University of Missouri, for whom it was prepared and tested by the Gilliland Laboratories, Marietta, Pennsylvania. The toxin, of necessity, differed from the usual preparations, since it was devoid of preservatives added to prevent bacterial growth. The presence of the preservative would doubtless have had an effect upon the growth of paramecia, and consequently would have complicated the study. The toxin used throughout the study had an M. L. D. of 300, i.e., $\frac{1}{300}$ cc. was the minimum amount required to kill a guinea-pig weighing 250 grams in four days.

The experimental animals were in each case grown from day to day in 0.2 per cent timothy-hay infusion to which measured quantities of toxin had been added. The timothy-hay infusion was prepared as has been previously described and enough was made at one time to last throughout the experiment. This infusion after sterilization had a pH of 7.32. The medium for each day was prepared by adding 0.12 cc. of the diphtheria toxin to 20 cc. of the sterile hay infusion. For the growth of each animal 0.5 cc. of this mixture was added to the cavity of a culture slide in a Petri dish and 0.2 cc. of a twenty-four-hour mixed-bacterial culture in hay infusion was added for food. Thus there was provided in each cavity of a culture slide for each paramecium a total of $\frac{1}{333}$ cc. of diphtheria toxin—almost a minimal lethal dose for a guinea-pig weighing 250 grams. It was found that when a greater amount of toxin was added the presence of the nutritive materials in the added broth containing the toxin brought about the development in the hay infusion of bacterial scums and precipitates that hindered the normal growth of the paramecia and complicated the experiment by increasing the possibility of the active toxin being modified or destroyed by the numerous bacteria and their products. It was intended to provide in the medium the maximum amount of diphtheria toxin that could be used without causing the formation of such bacteriological scums and precipitates. After many trials it was found that the mixture of 0.12 cc. of toxin to 20 cc. of the hay water was the most satisfactory.

That detoxification did not occur, when diphtheria toxin was added to the hay water, was determined by injecting guinea-pigs with fatal doses of the toxin diluted in 0.2 per cent hay infusion. That the presence of bacteria added to the hay infusion-toxin mixtures did not modify or destroy the toxin is indicated by the following experiment. On August 30th, a guinea-pig weighing 380 grams was injected with 1.5 cc. of a hay infusion-toxin mixture. This time 0.12 cc. of the toxin was added to 10 cc. of sterile 0.2 per cent hay infusion, and the mixture was then heavily inoculated with air bacteria and inoculated at 25°C. for six hours. At the end of this time, the contaminated hay infusion-toxin mixture was passed through a Berkefeld filter and 1.5 cc. of the sterile filtrate injected intraperitoneally into the guinea-pig. The animal died in forty-eight hours. Two control animals each injected with filtrate similar in every way to that described above, except that the toxin had been omitted, remained alive.

Pure lines of three species of paramecia were used in this study. The pure line of P. caudatum came from an individual isolated from an aquarium in Columbia, Missouri, on August 7, 1926, and the lines of P. aurelia and P. calkinsi from individuals isolated from Woodruff's pedigreed stocks.

In each experiment undertaken one animal was isolated and its immediate descendants were used as the beginning of, a) four lines to be grown from day to day in cultures containing diphtheria toxin; b) four lines in cultures containing boiled toxin, and, c) four lines in cultures containing no toxin. As usual, the daily isolation method was used and records were kept of the number of divisions per day. Hay infusion-toxin mixtures were made each day just before the transfer of animals to fresh media. The temperature of all infusions containing paramecia was controlled to between 23° and 25°C., since the infusion-toxin mixtures at higher temperatures often developed troublesome scums and precipitates.

It was found that lines introduced into cultures containing toxin as well as into cultures containing boiled toxin did not

survive many days. Wherever a line died out in such a culture, a new line was started to take its place. Such new lines were in every case started from individuals discarded from the control lines growing in the culture containing no toxin. Thus, while there were only four lines under observation for each kind of media at any one time, the total number of lines used during the entire time of the experiment in the culture that contained toxin and in the cultures that contained boiled toxin was greater than four. At the end of each experiment it was possible to determine for each type of medium used the total number of lines introduced and the average duration of life in each line.

TABLE 13

Effect of diphtheria toxin on lines of P. caudatum. Duration of experiment, sixteen days

	TOTAL NUMBER OF LINES INTRODUCED INTO THE MEDIUM	AVERAGE NUMBER OF DAYS LINES SURVIVED	PERCENTAGE OF DEATHS	MEAN DIVISION RATE
A. Medium containing toxin	9	8.3	10	1.08
B. Medium containing boiled toxin	7	8.0	8	1.17
C. Medium containing no toxin	4	16.0	1	.73

The data obtained with respect to P. caudatum are shown in table 13. It is evident from a consideration of the items in the table that, under the conditions of the experiment, diphtheria toxin had no appreciable effect upon this species of paramecium. The average number of days the lines (A) survived in the presence of the toxin was 8.3, as compared with 8.0 for the lines (B) growing in the presence of boiled toxin. The mean division rate for the lines (A) growing in the presence of toxin was 1.08, as compared with 1.17 for lines (B) growing in the presence of boiled toxin. There was, similarly, no difference of any significance in the percentage of deaths in the lines under the two experimental conditions, since it was 10 per cent for the former and 8 per cent for the latter.

The death rate and division rate in the four lines in (C) that were grown in the media containing no toxin is quite different from that for the lines in (A) and (B). This difference is due to the absence in (C) of certain nutritive substances which were present in the toxin added to all media used for lines in (A) and (B). These nutritive substances include peptone, beef extract, and dextrose in small quantities.

TABLE 14

Effect of diphtheria toxin on lines of P. aurelia. The experiment covered a period of nineteen days, from August 11 to August 30, 1926. Twelve sister lines of paramecia were started in the infusions, four in A, four in B, and four in C. In each case where a line died out another was started to take its place. Such replacements in every case were made from animals discarded from line C. Each replacement thus made constituted the beginning of a new line and the total number of lines introduced into each medium throughout the time of the experiment is shown in the first column

	TOTAL NUMBER OF LINES INTRODUCED INTO MEDIUM	AVERAGE NUMBER OF DAYS LINES SURVIVED	PROBABLE ERROR	PERCENTAGE OF DEATHS	MEAN DIVISION RATE	PROBABLE ERROR
A. Medium containing toxin	13	5.8	3.5	13	1.0	.2
B. Medium containing boiled toxin	7	10.0	3.6	6	1.0	.2
C. Medium containing no toxin	5	16.5	1.6	4	.9	.5

The results for P. aurelia, as shown in table 14, might seem to indicate that the diphtheria toxin had some effect upon metabolism in this species. However, when one considers the large probable error for some of the figures in the table it becomes evident that the conclusion may also be made here that the presence of the toxin had no appreciable effect upon the division rate or upon the time that a race of these animals is able to survive in the infusions. It is particularly noticeable that the percentage of deaths in the lines of P. aurelia growing in the presence of a toxin was 13 as compared with a percentage of 6 for the lines in the presence

of the boiled toxin. Here it might seem that the presence of the toxin had some influence upon the death rate in the lines of P. aurelia. But the results obtained with P. caudatum, as shown in table 13, and with P. calkinsi, as shown in table 15, do not support this conclusion.

The results of a study of diphtheria toxin upon lines of P. calkinsi, as shown in table 15, justifies the same conclusion as with P. caudatum and P. aurelia, that the toxin had no appreciable effect upon the division or death rate of this species.

TABLE 15

Effect of diphtheria toxin on lines of Paramecium calkinsi. The experiment covered a period of twenty-four days, from August 6 to August 30, 1926. Twelve sister lines of paramecia were started in the infusions, four in A, four in B, and four in C. In each case where a line died out another line was started to take its place. Such replacements were, in every case, made from animals discarded from line C. Each replacement thus made constituted the beginning of a new line, and the total number of lines introduced into each medium is shown in the first column of the table

	TOTAL NUMBER OF LINES INTRODUCED INTO MEDIUM	AVERAGE NUMBER OF DAYS LINES SURVIVED	PROBABLE ERROR	PERCENTAGE OF DEATHS	MEAN DIVISION RATE
A. Medium containing toxin	7	13	6	4	.6
B. Medium containing boiled toxin	5	18	5	3	.6
C. Medium containing no toxin	4	23	.8	1	1.5

The results here obtained in the study of the relation of diphtheria toxin to growth in paramecia are similar to those obtained by Oehler ('21) on Colpidium. He grew three species of this organism in pure diluted bouillon cultures of Bacillus diphtheria. He worked with mass cultures of the protozoa and was not able to find any effects produced by the diphtheria cultures which were not produced in cultures of non-pathogenic bacteria. He was concerned, however, with cytological effects and not with effects upon the division rate.

DISCUSSION

It has been shown in the foregoing study that races of paramecia are able to adapt themselves to growth in toxic cultures of Bacillus pyocyaneus. The evidence indicates that this adaptation is an immunization to the soluble pyocyaneus toxin, although it is not established beyond question that it is not a case of acquired tolerance to some other deleterious substance. Whatever may be the fact as between the development of immunity or of tolerance, the adjustment of races of paramecia to growth in the presence of deleterious substances, as described in this investigation, differs from most studies that have been made of acclimatization in unicellular organisms, because the daily-isolation method has been used throughout. This eliminates the agency of selection. It is only by this method that one can hope to demonstrate the development in races of protozoa of either acquired tolerance or immunity as distinguished from the survival of tolerant strains that have originated in the mass independently of environmental influences.

In an attempt to demonstrate a true case of immunization in protozoa by growing the animals in cultures of toxin-producing bacteria, one is never sure that the deleterious effects of all toxic agents other than the 'toxin' have been eliminated. A better method of demonstrating a case of immunization in protozoa would be to find some standardized and purified toxin that affects paramecia and to add it in measured amounts to non-toxic hay infusions in which the protozoa grow. In the present investigation this was attempted only in the case of diphtheria toxin which was found not to have any appreciable effect upon paramecia.

The present study shows that paramecia may be grown in pure cultures of B. pyocyaneus. Even though it may be unwise to use any pathogenic bacillus extensively, the evidence here presented shows that paramecia may be grown in pure cultures of at least one species of bacteria. It is reasonable to hope that further investigation will reveal other species of bacteria, some of which may be non-pathogenic, that will be as serviceable in this respect as B. pyocyaneus.

SUMMARY

1. Cultures of Bacillus pyocyaneus are toxic under certain conditions to Paramecium aurelia, P. calkinsi, and P. caudatum, but pure-line cultures of these three species of paramecia may acquire a tolerance for this toxic agent.

2. Races of paramecia may be grown for long periods of time in pure cultures of B. pyocyaneus.

3. The data presented indicate that the toxic agent in cultures of B. pyocyaneus that is lethal to paramecia is probably the soluble toxin known to be produced by this bacillus.

4. Hydrocyanic acid was found in measurable quantities in hay-infusion cultures of B. pyocyaneus, but not in sufficient quantities to be injurious to paramecia.

5. Hay-infusion cultures of Bacillus enteritidis were lethal to paramecia. All attempts to develop tolerance in paramecia for the toxic agent in these cultures failed.

6. Under the experimental conditions that prevailed, diphtheria toxin had no appreciable effect upon the division rate or death rate of three species of paramecia.

7. The reliability of the method of washing paramecia until bacteria-free and of establishing and growing races of them in pure cultures of bacteria is discussed.

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THE CHROMOSOMES OF THE GUINEA-PIG[1]

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TWO PLATES (TWENTY-FOUR FIGURES)

AUTHOR'S ABSTRACT

The spermatogenesis of five guinea-pigs was studied. The spermatogonial chromosome number is approximately sixty-two plus or minus two. The primary spermatocyte number is approximately thirty-one. The spermatogonial number in the early prophase is lower than it is in later stages. This condition is due to late fragmentation of the large chromosomes found in the earlier stage. A possible sex chromosome of the X-Y type may be identified. Its components segregate during the first maturation division.

The present investigation is the third of a series of studies being carried on at this laboratory dealing with the chromosomes of the common rodents. The practical genetical application of these studies and the broader theoretical aspects have been discussed by Painter ('26) in the first of this series of papers. The guinea-pig differs from the other rodents so far investigated in its very high chromosome number. If my observations have been correctly interpreted, this high number is, however, a late phylogenetic acquisition and is brought about by a fragmentation of larger chromosomes, as will be shown below.[2]

The first extensive work dealing with the chromosomes of guinea-pigs was that of Stevens ('11), in which she gave the diploid number as approximately fifty-six and the sex chromosomes as of the X-Y type. Recently, Harmon and Root ('26) have described the diploid number as thirty-eight, but agree that the sex chromosomes are of the X-Y type.

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[2] The essential results of the present paper were given in a brief note (by Painter). Science, vol. 64, p. 336, 1926.

Five mature male guinea-pigs were used for this study. After the animals were stunned by a blow on the head, the testes were removed, cut into small pieces, and the tubules teased apart very quickly and fixed in Allen's modification of Bouin's fluid. The tissue was subsequently treated according to the technique outlined by Painter ('24). Sections cut at 8 μ were found to be most satisfactory. Divisions of all orders were numerous in the testes of adult guinea-pigs.

Six equatorial plate views of dividing spermatogonial cells are given (figs. 1 to 6). The chromosome counts in these cells vary slightly, the number identified being sixty-two (figs. 3 and 6), sixty-three (fig. 4), and sixty-four (figs. 1, 2, and 5). A number of other spermatogonial plates were counted, giving essentially the same results as in the accompanying figures. In no case was the number found to be less than sixty or greater than sixty-four.

There are few spermatogonial chromosomes of striking form or size. Two chromosomes, labeled *a* in the drawings are sometimes conspicuous because of their great size. Undoubtedly, they are the large chromosomes identified by Miss Stevens. The remaining elements are much smaller and range in shape from bent rods to somewhat oblong blocks. The presence of such a large number of small elements makes counting difficult and may account in part for the apparent variation in numbers observed.

In late prophases of dividing spermatogonia a very interesting condition is encountered (figs. 7 and 9). (For the sake of clearness some of the chromosomes in these cells have been omitted in the figures.) The chromosome number at this time is clearly lower than in the equatorial plate stage. Although the exact number has not been determined, due to confusion with regard to the interpretation of transition stages in chromosomes, it is approximately forty. A close study of individual chromosomes reveals one or more cross

constrictions in many of the elements (figs. 7 and 9). Later, these constrictions break through, but the fragments are held together by chromatic strands and give somewhat the appearance of a string of sausages. In figure 7d and figure 9e this process is especially clearly shown. Subsequently, these chromosome segments apparently separate entirely, for there is no visible connection between the elements in later stages and little or no suggestion of the arrangement found in prophases. By the equatorial plate stage, the number is clearly over sixty.

PRIMARY SPERMATOCYTES

During the early growth period an acidophilic element and a chromosome nucleolus are associated together (figs. 22, 23), but, as in the case of the mouse (Gutherz, '22; Cox, '26), these components separate later, the acidophilic body disappearing and the chromosome nucleolus persisting and entering the first maturation spindle.

In equatorial plate views the elements are well separated (figs. 10 to 13 and 16). Counts made at this time show that at least thirty separate elements are present (figs. 11, 12, and 16), and in the clearest cells thirty-one elements are found (figs. 10 and 13). The latter number is regarded as probably the correct reduced number.

First maturation spindles viewed from the side are interesting for, frequently, one can easily make an approximate count of the haploid number of elements. In figure 14, for example, at least twenty-nine elements can be recognized. (This figure and the one following are 'spindle dissections' in which the individual elements are separated.) A number of similar counts have made it certain that the elements seen in the equatorial plate view have been correctly interpreted. One tetrad, decidedly larger than the rest of the chromosomes (labeled a in figs. 12 and 16), has obviously been derived from the pair of large spermatogonial chromosomes, as Stevens, and Harmon and Root have shown. Frequently, in telophase stages of this division, one large element lags behind in the

spindles, and then as division occurs it apparently breaks up into segments, suggesting that we are dealing with a compound chromosome (figs. 18, 19). This condition may be interpreted as an explanation of the apparent discrepancy between the possible diploid number of sixty-four and the haploid number of thirty-one. As in the case of the spermatogonial elements, most of the tetrads are much alike in form and size.

It has not been possible to identify with any degree of certainty a sex chromosome in the earlier phases of the first maturation spindle, although here and there cells are found in which one element is suggestive of an X-Y complex. In telophase stages it is not unusual to observe, lagging in the spindle, two bodies, one of which is large and the other small (figs. 17, 20, 21, and 24). Both Stevens, and Harmon and Root have observed this structure and have identified it as being made up of a large X and a small Y component. Stevens describes the separation of these elements as coming either early or late. The figures indicate that the X and Y are segregated in this division just as in the case of other mammals (Painter, '24).

SECONDARY SPERMATOCYTE DIVISIONS

Few secondary spermatocytes were found in the material studied, and these were not very favorable for counting. One or two counts were made in which not less than thirty elements could be distinguished.

DISCUSSION

The present work has shown that the chromosome number in the guinea-pigs studied is very high and may be safely taken as sixty-two plus or minus two. Sixty-two is the average of the spermatogonial counts, which ranged from sixty to sixty-four, and thirty-one is the haploid number most frequently observed. It is not implied, of course, that there is really any variation in the chromosome number in the germ cells, but the small size of the cells, and the small size of many of the chromosomes with the attendant absence of distinctive

shapes, greatly increases the difficulty of counting and, consequently, the liability to error. Considering the nature of the material, the writer is probably not justified in fixing the number within closer limits than sixty-two plus or minus two. These results are in general accord with the work of Miss Stevens, who reported approximately fifty-six as the diploid chromosome number. On the other hand, my results are in marked disagreement with the low number of thirty-eight reported recently by Harmon and Root.[3]

The lower chromosome number of Harmon and Root may, perhaps, be accounted for in part through the use of a technique which has not entirely prevented the tendency for adjacent elements to coalesce. Experience at this laboratory has shown that the method used by them (cold Flemming) is not quite as favorable for mammalian chromosome study as the modified Bouin technique. Thus, in figure 6 of their work, which is a polar view of the first maturation division, they have counted nineteen elements, many of which are obviously compound in nature. Normally preserved mammalian tetrads appear much as bivalent elements do in insect spermatogenesis and do not show the tendency to throw off buds, such as we see in figure 6 of Harmon and Root. In view of the counts made in the present study (figs. 10 to 13 and 16) it seems probable that, in the material of Harmon and Root, some adjacent tetrads have fused together, forming compound masses which they have interpreted as one bivalent chromosome. As the writer interprets figure 6 of Harmon and Root, there are about thirty elements present, a number which is very close to the haploid count of the present work.

The spermatogonial count of Harmon and Root, as shown in figure 2 of their paper, cannot be accounted for on the basis of inadequate preservation, but is due in large measure to another cause. The present work shows that in prophase stages of spermatogonia, the chromosome number is much lower than in fully formed spindles and that many of these

[3] For review of earlier work see Harmon and Root (l. c.).

larger elements break up into smaller bodies (figs. 7 and 9) before the equatorial plate stage. Figure 2 of Harmon and Root is clearly a cell in which this breaking up of the chromosomes has not yet taken place. Close observation of the individual elements reveals a number of constrictions in the chromosomes, indicating that this breaking-up process was under way, and perhaps was masked by the technique employed. It is possible that in the race of guinea-pigs employed by Harmon and Root the chromosomes do not break up in the spermatogonial metaphases, as they do in the Texas strain.

All recent workers on spermatogenesis in guinea-pigs have observed in the first maturation division a lagging element apparently made up of a large and a small part. This structure has been interpreted as an X-Y sex chromosome complex, and the probabilities are that this interpretation is correct. However, it should be realized that the evidence for this conclusion is neither critical nor complete, but rests largely on the similarity of this element to the X-Y sex chromosomes of the other mammals and of the insects.

The most interesting feature of the present work is the high chromosome number of the guinea-pig as compared to the other rodents and to mammals in general, and the method by means of which this high number is derived from a relatively simple condition. In all the other rodents studied in this laboratory (mouse, rat, rabbit, and squirrel[4]) the chromosome number ranges from forty to forty-four. In view of the work on insects, especially that of McClung and his students on Orthoptera, we should expect that, within small groups such as the rodents, there would be a close similarity in the chromosomes, both as to number and morphology. If the interpretation given to my observations is correct, this conclusion is essentially true in the present case, the guinea-pig having originally possessed the typical rodent complex. This number later has broken up into the larger number of smaller more uniform elements.

[4] The results of the study of the squirrel are as yet unpublished.

From the standpoint of the geneticist, in view of the large number of tetrads, linkage groups should be much rarer in the guinea-pig than in the other rodents. This conclusion is based upon the supposition that the change has been due solely to fragmentation of the chromosomes, and not to any important rearrangement within the elements themselves or to the acquisition of new elements.

SUMMARY

1. The spermatogonial number is approximately sixty-two plus or minus two.

2. The primary spermatocyte number is approximately thirty-one.

3. The spermatogonial number in the early prophase is lower than it is in later stages. This condition is due to late fragmentation of the large chromosomes found in the earlier stage.

4. A possible sex chromosome of the X-Y type may be identified. Its components segregate during the first maturation division.

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PLATE 1

Figures 1 to 6 represent spermatogonial cells of the guinea-pig.

1, 2, and 5 Equatorial plate stage, showing sixty-four chromosomes.

3 Equatorial plate stage, showing sixty-two chromosomes.

4 Equatorial plate stage, showing sixty-three chromosomes.

6 Prophase stage, showing sixty-two chromosomes.

7 and 9 Parts of early prophase stages, showing fragmenting of chromosomes.

8 Side view of a primary spermatocyte spindle with division of a possible X-Y tetrad.

10 Late diakinesis with thirty-one tetrads.

11 and 12 Polar views of equatorial plate stages of primary spermatocytes, showing thirty tetrads.

13   Late diakinesis stage, showing thirty-one tetrads.

14 and 15   'Spindle dissections' of side views of primary spermatocyte spindles.

14a and 15a   Large tetrad.

16   Polar view of equatorial plate stage of primary spermatocyte, showing thirty tetrads.

17, 20, 21, and 24   Side views of primary spermatocyte spindles with division of possible X-Y elements.

18 and 19   Side views of primary spermatocyte spindles, showing the octad dividing into four chromosomes.

22 and 23   Growth stages, showing separation of acidophilic and basophilic elements in heterochromosomes.
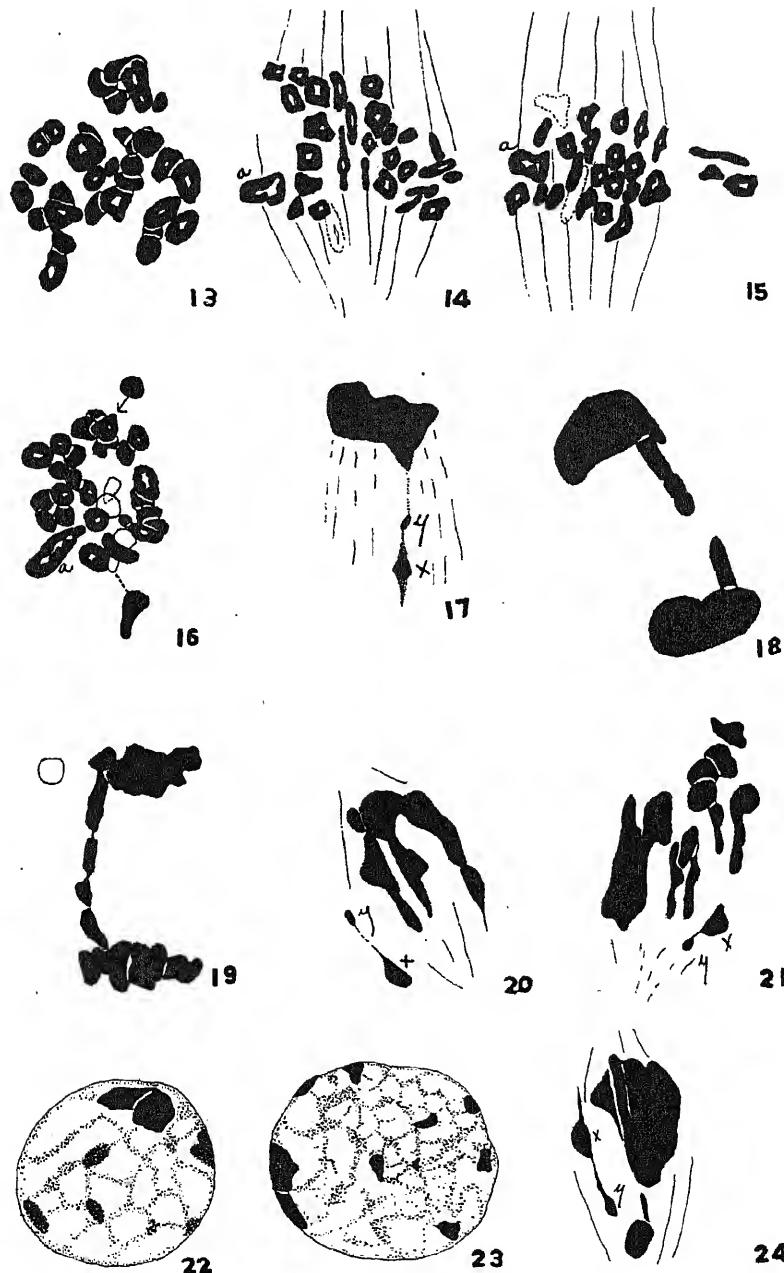
THE BREEDING SEASON OF THE OPOSSUM (DIDELPHIS VIRGINIANA) AND THE RATE OF INTRA-UTERINE AND POSTNATAL DEVELOPMENT[1]

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NINE CHARTS AND FOUR HELIOTYPE PLATES

AUTHOR'S ABSTRACT

The breeding season of the opossum at Austin, Texas, begins in January, following a three months' anoestrous period. The modal point for ovulation days is, reached in the third week. The rate of intra-uterine development was investigated chiefly by surgical removal of one uterus, noting the stage attained by the ova therein and allowing the surviving uterus to incubate its ova a precalculated period of time. Unique charts epitomize the results. The primitive-streak stage is completed, the medullary groove and chorda begin at seven and one-half days post coitum, seven days post ovulationem, leaving only five and one-half days' actual development of the embryo to birth. The rate of development is compared with Eutherian mammals.

The curve of postnatal growth has the shape of embryonic growth curve of higher mammals. The eyes and lips open at about fifty days (young the size of mice). At this time the young leave the teat for the first time, but are not weaned for about thirty days more. Soon after weaning, the mother may become pregnant again. At ninety to one hundred days (young size of large rats), the young may begin to shift for themselves.

Since the study of the early development of the opossum was begun about 1913, protocols of over 800 females of D. virginiana have accumulated. This number includes, of course, every animal that was received, whether it contributed data of value or not. The animals were collected for other than biometrical purposes; hence the percentage of cases furnishing usable data of a statistical nature for the present paper is proportionately small. Nevertheless, a

[1] The work on the opossum was begun at the suggestion of Dr. J. T. Patterson, Professor of Zoölogy, the University of Texas, about 1913. It was prosecuted intensively through the generous financial aid and moral support of The Wistar Institute, Dr. M. J. Greenman, Director, and with the assistance of Dr. C. H. Heuser, then fellow of The Wistar Institute. It is to the skill of Doctor Heuser that most of the photographs presented in the four plates accompanying this article and former papers of this series are due. Some of the photographs were taken from fresh living material in January and February of 1917 at Austin, Texas. The embryological investigations soon gave way in large measure to physiological studies in which the following generously aided: Mr. H. A. Wroe,

survey of the protocols yielded a sufficient amount of material
to make its collation and publication seem desirable. These
data, together with the writer's previous studies on the
oestrous cycle of the opossum, should enable the embryologist
and the physiologist to proceed in the collection and classi-
fication of material without the loss of time involved in
pioneering.

This conclusion as to the extent of the data to be presented
seems all the more justified when one considers the disap-
pointing dearth of similar data concerning other mammals,
even the laboratory rodents and the domestic animals that
have been so much used for embryological studies. Thus,
for example, of very few species do we know the stage at-
tained at any given number of hours or days with any cer-
tainty. They can be counted on the fingers of one hand:
mouse, rat, rabbit, guinea-pig, and domestic pig; and the data
even in these forms are far from satisfactory. The opossum
is, furthermore, the first wild form in which any considerable
information as to its reproductive processes has been worked
out. That it is also a marsupial, America's only pouched ani-
mal, lends further interest to the material under discussion.

The breeding season will be outlined in part I; part II
will be devoted to the main topic, the intra-uterine develop-
ment; part III of the postnatal growth.

I. THE BREEDING SEASON

In 1923, I outlined the breeding season of the opossum in
the following words:

The breeding season of the Virginia opossum begins in January
at Austin, Texas, and probably several weeks later in the North.

A few individuals may come into heat in the first week of the year, but more enter this condition in the second week. In the third week the season is at its height; hence the embryologist desirous of securing eggs and embryos would best time his collection during the last week in January and the first week in February. The prevailing weather seems to have no effect on the onset of the breeding season.

By the middle of February most females captured have young in the pouch. But late in the spring and the summer there is great irregularity in the condition of the females, so that one may capture females with small young in the pouch any time between May and September. Many reasons may be advanced for this variability: accidents to the mother resulting in the early loss of the young; variability in weaning age; condition of the female, the robust reproducing faster than the weak; age of animals, the very youngest 'yearlings' and the oldest multiparae being the latest to come into heat (p. 352).

After studying the available data more intensively, I find these statements need but slight correction. They may now be amplified by further analysis of the old and additional details ascertained since 1923.

The progress of the breeding season for the first half of the year may be visualized from text figure A, in which the litters of pouch young are represented by black dots or discs, graduated in size roughly according to the size of the pouch young. Thus the largest circles represent pouch young about ready to leave the mother; the next size are weaned young still under the care of the mother; the third size are sucklings, but detached from the teats, and so on, to the smallest dots which represent young a week or less old. The absolute number of litters has no meaning, except for the early part of the year, since collection of animals by purchase was most brisk in January, February, and March, slackened greatly in April, and was accidental and sporadic after that. Only two years were any observations made in the summer, for vacation usually put an end to the field work until late in the fall.

An effort was made, by searching through the records of the United States Biological Survey, to determine the beginning of the breeding season in States farther North. In spite of the numerous records on file, an insufficient number of usable cases was found upon which to base safe deductions.

I leave this to future workers. The reason that most of the records are not useful for the present purpose is that the size of the pouch young is seldom indicated.

The opossum has no true hibernating season, but certainly an anoestrous period in the early winter. No pouch young, large or small, were ever taken in October, November, or December. In one case, ripe follicles were encountered in December (female no. 16, 455 mm., December 6, 1915). In one case, pouch young were born in the first week of January; in three cases, in the second week (text fig. B), hence these four exceptions constitute the only approaches to ovulation in December. All other females captured in October, Novem-
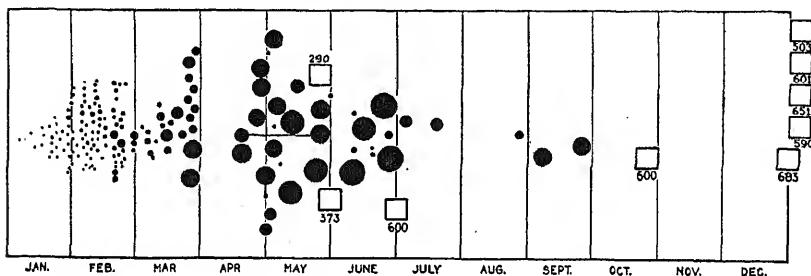
Text fig. A Chart to illustrate the progress of the breeding season. Each dot or disc represents a litter of pouch young, the size of the dot roughly corresponding to the size of the young (see text). Squares represent individual animals, with weight in grams (appendix D).

ber, and December were in the 'resting' condition absolutely, and usually no trace of a corpus luteum was to be found in the ovaries. Most females are in anoestrum in the first week of January also (text fig. B), hence the anoestrous period must be at least three months in length. It is of importance to note this point in endeavoring to determine whether the opossum bears two or three litters in a year.

The male seems to possess spermatozoa during the entire year. Painter ('22), however, found many spermatogonial, but few maturation divisions at the end of the calendar year and more maturation divisions in January. It would be interesting to learn more concerning the reproductive habits of the male opossum. It is quite probable that the male is capa-

ble of fertile coitus even though active spermatogenesis be in abeyance over several months, for the spermatozoa would remain viable in the epididymis over this period. Thus in bats the germinal epithelium of the testes undergoes atrophy during hibernation; however, in some bats, the epididymis is also involved in the general atrophy and is devoid of sperms (Nyctinomus mexicanus); in others the epididymis carries motile sperms all winter (Myotis sp.). There doubtless is
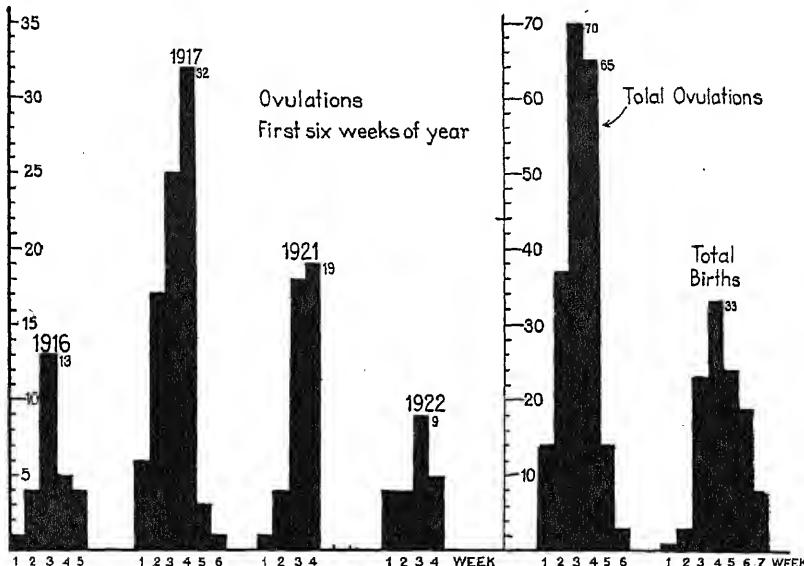
Text fig. B Ovulation dates and birth dates plotted according to the week following January 1 of the calendar year for certain years, and totals for all years.

every gradation among mammals from almost complete anoestrous atrophy of the testes and accessory sex glands to continuous and unvaried sexual potency, as in primates.

The problem of determining the opening of the breeding season in January may be approached in another way. Since oestrus and ovulation constitute the obvious signs of sexual activity, this date was determined in as many cases as possible. More could have been added with an approximation to the truth by estimating the dates with the aid of text figures C and D on the rate of development. But this was

This is a full-page illustration rotated sideways: a chart titled "Selenka's Cases. (1887)" with a grid showing developmental stages across days 1–13. The vertical axis is labeled "Copulation observed" with case numbers: 190, 134, 314, 114, 298, 838, 343, 194, 306, 299, 203, 173, 320, 293. The horizontal axis shows "Days" 1 through 13. Annotations include "360", "585", and "?".

148

C₁

Text fig. C Chart 1, to show rate of development from one stage to another, both stages taken from the same female, the intervening intervals of time controlled to the minute. (For details see text, part II.) The numbers are the protocol numbers of the animals furnishing the eggs and embryos.

C₂

Days: 1 2 3 4 5 6 7 8 9 10 11 12 13

Degenerating Ova: 566 521 837 762 798

841 (16)
561 (16)
337 (16)
969 (16)
336 (25)
342 (28)
193
191 292
344
544
927
349
356
931

149

avoided for the reason that the results were to be used, if possible, to check the correctness of this chart itself. For it seemed reasonable that if a set of birth dates, based on an entirely different lot of material, could be prepared in addition to ovulation dates, then by calculation of the mean and the standard deviation in each of the distributions, the period of gestation (ovulation to birth) might be deduced by dif-
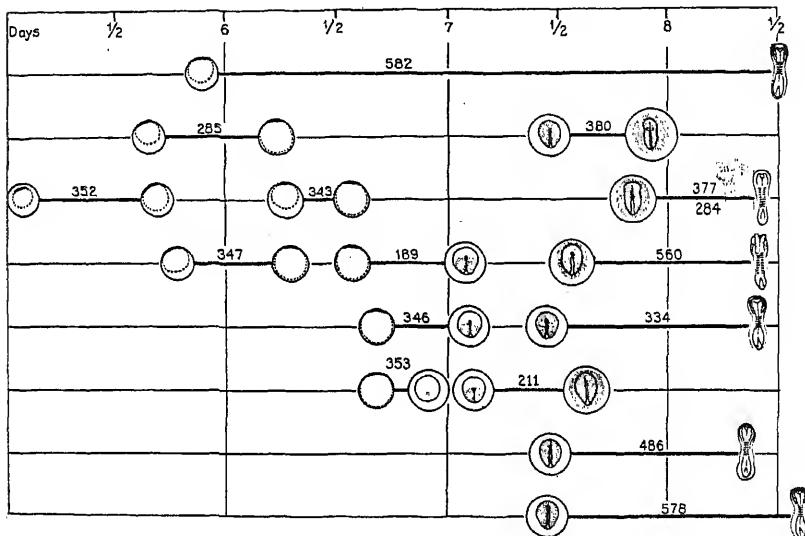
Text fig. D   Chart 2. Similar to text figure C, shorter intervals involved. The scale is double that of text figure C, hence cannot be imposed upon the latter. The interested reader could photograph this figure × ½ and fit the photograph into its place in text figure C.

ference of the means, and the significance of this difference might be judged in the light of the variation of the distributions (v.i.). These two sets of data were prepared, 110 dates of birth and 203 of ovulation (table 1). They are grouped by weeks of the year. For the ovulation dates only those cases are used in which actual observation determined the date absolutely and those which fell, by calculation, near the middle of the week; the border-line cases were discarded. It is thought that in this way the warping of the data by

preconceived ideas on rate of development would be entirely avoided. The dates of birth were either determined by actual observation (some twenty cases), the remainder by the aid of the graphs of growth rate shown in text figures E and F,

TABLE 1

Ovulation and birth dates, by years and for the first six weeks of each year (compare graphs in text fig. B)

YEAR	WEEK	JAN. 1–7 1	8–14 2	15–21 3	22–28 4	29–FEB. 4 5	5–11 6	12–18 7	TOTAL
1914	Ovulations			1					1
	Births								0
1915	Ovulations	1	8	3	3	5	1	0	21
	Births								0
1916	Ovulations	1	4	13	5	4	0	0	27
	Births	0	1	1	0	4	2	4	12
1917	Ovulations	6	17	25	32	3	0	0	84
	Births	0	1	0	7	4	3	1	16
1920	Ovulations	1							1
	Births				8	3	5	1	17
1921	Ovulations	1	4	18	19				42
	Births	0	1	4	5	7	4	2	22
1922	Ovulations	4	4	9	5				22
	Births	0	0	8	0	3			11
1924	Ovulations				1	1	1		3
	Births								0
1925	Ovulations					1	1		2
	Births								0
Totals	Ovulations	14	37	70	65	14	3	0	203
	Births	1	3	23	33	24	19	8	111

which are probably quite accurate. All of the figures are given in table 1. The figures for the years 1916, 1917, 1921, and 1922 are expressed in the form of graphs shown in text figure B, which also contains graphs for all of the data for all years—one for the ovulation dates, one for the birth dates.

An inspection of the graphs shows that the third week of the year has the advantage in number of ovulations, though the fourth week is not far behind. After the fourth week, nearly all females brought in from the field may be expected to be pregnant or to carry pouch young. To secure the optimum results, the embryologist, desirous of securing early stages, should begin collecting not later than the end of the second week of the calendar year.

Reduced to figures, the means, or, mechanically speaking, the centers of gravity, of the four annual charts shown in text figure B, as calculated for me by Dr. H. L. Dunn, are as follows:

$$1916, \; 26.315 \pm 0.912 \; days$$
$$1917, \; 25.259 \pm 0.521 \; days$$
$$1921, \; 26.667 \pm 0.539 \; days$$
$$1922, \; 21.636 \pm 1.162 \; days$$

The figures argue for variations from year to year. Thus 1922 appears to be about five days earlier than the other three. I could find no conditions of temperature or rainfall from the weather reports of the preceding years to account for the early opening of the breeding season in 1922. On the other hand, 1925 was very late. In that year not one case of ovulation was encountered in the first two weeks, although a sufficient number of animals was on hand to make the observations significant. The peak came in the fifth week. Now, in central Texas, this collecting season was preceded by a severe drought which, acting through the lack of food, may have caused the delay in the accumulation of the sexual hormones at that season.

NUMBER OF LITTERS A YEAR

Barton ('23) and Meigs ('47) were both of the opinion that the opossum brought forth two litters a year, the first in January and the second in May. Audubon and Bachmann ('51) considered March, May, and July to be the dates for the three litters in the latitude of South Carolina. The latter, however, suggest (p. 124) that the July brood does not repro-

duce until the following May. But since these authors seem to be wrong about all of their calculations on the development of the opossum (v.i.), I should place no reliance upon their opinion on the breeding season.

There can be no doubt that there are at least two litters a year, for there are enough records to show that when one litter is reared another gestation follows (text fig. A). Hence it might at first be thought a matter of simple arithmetic; for if it takes three months to rear one brood (v.i.), three can be reared in nine months, leaving three months for the anoestrum. It is probable, however, that this is possible only to a few unusually vigorous or fecund individuals. If the time of the first oestrus of the year is variable (text fig. B), the time of the second must be much more so, since it depends upon a great many more factors: length of gestation, length of lactation, ovulation, and other physiological processes involved in reproduction in which there is great variability from one female to another. Hence, if one knew the facts and could construct ovulation charts for the early summer after the manner of text figure B, the curve would probably spread out over many weeks. Text figure A, meager as it is for May and June, seems to point in this direction.

An attempt was made to approach the problem statistically. In the lower portion of text figure F (v.i.) the weights of 192 females are plotted according to the nearest hundred grams weight. It is seen that there is a group of eleven females between 500 and 800 grams in weight; to these we may add the four cases around 900 grams. These fifteen females seem to be in a class rather sharply differentiated from the others. I suggest that these represent the August or September litters, the few third litters of the year. The details concerning these are given in appendix D. Nine of these cases are inserted as squares in text figure A. The 290-gram and the 373-gram females of June and the 600-gram female of July are either dwarfs, which is not probable, or exceptional late conceptions of the preceding year. To the same category belongs the litter of "two opossum young as large as small

kittens'' which Meigs found in February, 1845, presumably in the neighborhood of Philadelphia. On the other hand, numerous references of opossum young 'half grown' in the records of the Biological Survey range, so far as I have seen, from May to October, as is to be expected on the basis that breeding takes place only in the first half of the year. .

SUMMARY

The breeding season begins at Austin, Texas, with the calendar year following a three months' anoestrum, or period of reproductive rest, in October, November, and December. There is a slight variation from year to year, though for practical purposes, as, for example, for collecting embryos, the variation is unimportant and the information contained in text figure B is probably a sufficient guide.

There are at least two litters of opossum young a year in the Southern States. There is no unequivocal evidence of a third, but it seems probable that a third litter is produced by a small percentage of unusually fecund females. The opossum should be bred in captivity and individuals observed over a period of years. Such observations would not only fill the gaps left in our knowledge of the breeding season, but also complete my growth curve (v.i.), determine the age of puberty and settle other points which the two following sections still leave open.

II. THE INTRA-UTERINE DEVELOPMENT

The effort is made in this section to determine time relation as accurately as possible, so that a given stage may be secured by future workers with a reasonable degree of certainty. The chief method employed is that used by Bischoff on the rabbit ('42) and, more especially, on the dog ('45), and since then only by Selenka ('87) and myself. The method consists of removing successively two stages from the same animal, the interval between the two operations being controlled to the minute.

In the present work, the favorable animal was selected by mammary palpation, the left uterus was removed by aseptic surgery, and the stage of its contained ova noted. The animal was then allowed to live such a period of time as seemed necessary, from previous experience, to secure the desired stage from the surviving uterus, which thus served as Nature's own incubator for the contained ova. Since the ova of both uteri are always in the same stage of advancement, ovulation being simultaneous from both ovaries, the rate of development may be accurately gauged. That the operation has no deleterious effect upon the remaining ova, I have set forth in 1919 and determined by numerous experiments since.

The results of our studies upon the rate of intra-uterine development of the opossum are epitomized in text figures C and D. Text figure C gives the longer intervals between copulation and a given stage, or between two stages recovered from the same animal after the manner above described. This chart is supplemented by text figure D, which covers the cases in which only a few hours were allowed to intervene between the removal of the left and the right uterus. The sketches by which the stages are to be identified, though semidiagrammatic, were made by Mr. James Didusch from life or from photographs. They are faithfully drawn by him and should offer the reader an easy and reliable method of identifying the stages in question. Four plates of twenty-nine figures in heliotype contain typical stages for the further orientation of the reader. The arrows of the chart indicate the exact end points of the intervals; that is, the sketches are placed immediately to the right of the arrow point or the time when the second specimen was taken. The exceptions are lines 1 and 2 of text figure C where the figures of the newly born pouch young were drawn to the left of the arrow points so as to retain the sketches within the limits of the chart.

The sketches require some further words of explanation. As is well known, the ovum proper or 'vitellus' of the opossum is surrounded by a thick layer of albumen, so-called, and

a thin, tough shell membrane (Hartman, '19). The individual
ovum would therefore be represented by a dot in the center
of a ring; the two-celled stage, by two dots, etc. When more
than four blastomeres are present, it was found impracticable
to show them by dots; hence the actual number is indicated
by inserting the number itself. When the number of blasto-
meres has reached thirty-two, they are arranged in the form
of a hollow sphere without evident polarity; here the vitellus
is represented as a small circle in the center of the shell
membrane, as, e.g., nos. 314 and 173 shown in the three-day
column. Soon polarity begins to manifest itself by a thicken-
ing of the embryonic pole and a thinning out of the tropho-
blastic region (e.g., fig. 1), while the vesicle slowly grows in
diameter at the expense of the albumen (nos. 344 to 931).
The vesicle gradually encroaches upon the albumen by digest-
ing and utilizing it, and this process occurs faster at the
embryonic pole; hence there is left a shrinking crescent at
the lower pole of the egg, the disappearance of the albumen
being proportionate to the differentiation and to some extent
the growth of the egg as a whole (Hartman, '19, pl. 13). At
about the time the mesoderm begins to proliferate, the vesicle
now having attained the diameter of 1.5 mm., the albumen has
all but disappeared (no. 353, Hartman, '19, fig. 22, pl. 13). Up
to this period the egg is represented as in a vertical section;
from then on the surface view is shown as seen by transmitted
light. The primitive streak, Hensen's node, medullary
groove, outline of embryonic area, are shown by continuous
lines, the spread of the mesoderm by stippling. Progressively
larger circles indicate the growth of the ovum in diameter,
but not in exact proportion to such growth. For relative
sizes the reader is referred to the heliotype plates and the
protocols (Appendix A). For lack of space, too, embryos
only, without the chorionic vesicle, have been drawn from
the later somite stages on (about eight days). The later
embryos are represented in side view as contrasted with
the dorsal view of the earlier embryos. The degree of de-
velopment must be read off in certain landmarks, such as the
head, heart, limbs, mouth, eye, ear, and the general form.

SELENKA'S AND HILL'S DATA

The studies of Selenka comprise the only pertinent data in connection with the present paper, if one except certain few findings of Hill ('10) on Dasyurus, the Australian 'native cat.' It is important to study Selenka's material, for with the proper correction his data are useful in checking my own. He secured a hundred Virginia opossums and had them sent to his laboratory in Germany. The animals yielded some excellent stages well described and illustrated by masterly drawings which are clear and unmistakable. He or his helper witnessed a number of copulations, hence he is able to give the copulation age of a number of specimens, though he fails to give the exact protocols. On the basis of copulation age, Selenka's data fit in well with my own, and I have incorporated them in line 2, text figure C. The copulation age is derived by adding five days to the age stated by Selenka. Thus his '8-hour' stage is actually five days eight hours!

The reason for this is as follows:

Selenka estimated the time of the beginning of cleavage as exactly '5 times 24 hours'; and this estimate is based on a single batch of eggs which he mistakenly considered normal cleavage stages. I have previously shown this batch of eggs to be made up of fragmenting ova or unfragmented but degenerating ova, as in the case of his figure 1, Tafel XVII. All of the eggs are in such a condition as one expects to find about five days after ovulation (compare Smith, '23). One of the same batch of eggs had arrived at the '20-celled' stage, and Selenka thought this normal also, although he states that ova are always in the same stage of development in a given female, in which contention he is substantially correct. Curiously, nine of the eleven eggs of this batch were still in the tube and several are reported as having spermatozoa in the perivitelline space. This batch of eggs is consequently hard to interpret, for usually the opossum's ova reach the uterus in the unsegmented condition. A second batch of eggs exhibited the same wide range of variation as the first, which did not seem to perturb the author in the least. None

of them appears to be quite normal; the one shown by him in his figures 1 and 2, Tafel XVIII, for example, has the ear-marks of degeneration in the shrunken condition of the vesicle and its thick wall—a conclusion reached on the basis of numerous similar eggs in my possession. Selenka's earliest normal egg is therefore one which he considers ten hours old! (his fig. 3, Tafel XVIII). If five days be added to the ten hours, the time would be about correct, since it accords very well with my own no. 194 and fits well into the text figures C and D. We now know that ten hours is not too long an estimate for the performance of a single cleavage division, to say nothing of reaching the early bilaminar stage! Similarly, if five days are added to all of Selenka's estimates, the result is very nearly in accord with my own data, as may be seen by comparing Selenka's data (line 2, text fig. C) with the remainder of the chart. The sketches of line 2 are faithful copies from Selenka's own drawings.

In his studies of three Australian marsupials ('91), Selenka apparently estimates the age of the bilaminar vesicle, the primitive-streak stage, and the 6-somite embryo on the basis of the opossum previously studied, although he does not specifically so state. These three stages were removed fifteen, thirteen, and fourteen days, respectively, postcoitum. Similar variations are recorded by Hill ('10) in·the case of ova removed from Dasyurus viverrinus. He states: "I obtained unsegmented ova from the uteri 4, 5, 6, 7, and 8 days after coitus, 2-celled eggs 6 and 7 days after, 4-celled eggs 11 and 18 days after. In one case the young were born 8 days after the last observed act of coitus, in another, 16 days after and in yet another 20 days after" (footnote, p. 3).

Now these observations of Selenka and Hill lead Hill to state that in Dasyurus and apparently in other marsupials "a very variable period intervenes between coitus and ovula-tion," and that, as a consequence "the obtaining of any de-sired cleavage or early blastocyst stage is largely a matter of chance." Since the postovulatory interval, for which Hill and O'Donoghue ('13) coined the term 'postoestrum,' con-

stitutes an important element in the problem of the rate of
development in mammals, it demands more detailed discus-
sion.

THE POSTOESTROUS PERIOD

From the above-mentioned observations of Selenka and of
Hill a long postoestrous period would seem to be character-
istic of marsupials in general, as distinguished from the con-
dition in the Eutheria. On this point Marshall ('22) says:
"In the mouse, the rat and the guinea pig ovulation occurs
spontaneously during 'heat,' and generally, if not invariably,
during oestrus" (p. 130). The same is said to be true of the
dog and the ferret. Indeed, Marshall generalizes (l.c., p. 131)
as follows: "There can be little doubt that in the great
majority of mammals ovulation, as a general rule, occurs
regularly during oestrus"; and he cites the bat as an out-
standing exception. For in the bat it is now tacitly and gen-
erally accepted that mating takes place in the fall and con-
ception in the spring. This tradition has recently been called
in question (Hartman and Cuyler, '27), although we shall not
allow a discussion of the merits of this matter to detain us
here. Marshall's generalization, quoted above, needs revision
for the sake of exactness, for the fact is that ovulation does
not always occur during oestrus, but often follows oestrus
and copulation. In other words, there is often a postoestrum
in the Eutheria, albeit a short one. That this period is also
a short one in at least one marsupial is made probable, if
not established by more recent data on the opossum (Hart-
man, '24), as will be shown below.

That the data on marsupials are still insufficient for a
final disposition of the problem is not surprising, in view of
the fact that the relation of heat, copulation, and ovulation
both as to time and physiological cause in any mammal except
perhaps the rabbit is very unsatisfactorily known. Pertinent
facts of this kind are hard to gather from the literature for
any mammal, even the common laboratory mammals. For the
rat and the mouse the matter is not answered, despite the
attention that has been devoted to the embryology and physi-

ology of reproduction of these common laboratory rodents. An inspection of table 2, page 31, of Long and Evans' monographic study of the rat ('22) leads one to assume a delay of ovulation after oestrus, for this period is mostly past before the ova are very far down the oviduct. In the rabbit it has been known since Coste's beautiful work ('49) that ovulation occurs, without a great deal of variation, ten hours after coitus, and approximately the same thing is true for the guinea-pig (Loeb, '06). However, these two species differ from each other in' that the former has no well-defined oestrous period, but will accept the male and ovulate over wide periods of time. In the cat (Longley, '11) and the ferret (Robinson, '18), as in the rabbit, ovulation usually depends upon copulation and occurs a variable number of hours, usually many hours, later. In the case of the ferret, Marshall states that ovulation occurs during oestrus. The statements of Marshall and Robinson with reference to the ferret are, however, not mutually exclusive, for a female may clearly be in heat and copulate successively over considerable intervals, both before and after ovulation. Whether copulation and fertilization shorten the heat period or not is not known definitely for any mammal. In the cow there seems to be no such influence, according to Küpfer ('20) and Hammond ('27); in the rabbit a positive answer seems to be necessary and Loeb ('11) states the same for the guinea-pig. In both Dasyurus (Hill) and the opossum the mated female will soon fight off the male, which might indicate that copulation cuts short the oestrous period.

It is thus seen that the correlation of events surrounding the ovulation period needs further experimental study. The common procedure of workers in this field is to mate their animals at convenient hours of the day, regardless of whether the animals are at the beginning or the end of heat. This must be a source of considerable error. There is no other way than continuous observation night and day, so that both the first and the last signs of oestrus may be taken into account. Results of intensive experiment along these lines should yield useful results (v.s.).

Further data on the time of ovulation is given by Keller ('09) in the case of the dog, in which ovulation occurs in the latter part of oestrus; in my opinion, however, evidence presented in the paper is far from conclusive. Better evidence is presented by Corner ('22) that the sow ovulates regularly toward the end of oestrus. But in the cow, contrary to the opinion of Krupski ('17), whose observations were made on slaughter-house material and who places ovulation at the end of oestrus, both Murphy ('26) and McNutt ('26) note a delay of one or two days after the end of heat before ovulation takes place. Küpfer ('20) does not insist on a constant relation between heat and ovulation. The observations of Murphy and McNutt may be regarded as reliable because these workers noted the exact time of rupture of the follicle by their technique of daily palpation of the ovary through the wall of the rectum.

These few citations illustrate the paucity of reliable information on the presence or absence of a postoestrous period and the length of the period where it occurs. There doubtless is much variation from species to species and among individuals of a given species, but as yet there are no data upon which to calculate probabilities. What is clear, however, is that the method of dating embryos by copulation age is highly unsatisfactory, since this involves at least two variables, length of oestrus and of postoestrum, which in different cases may be added together or subtracted, which increases the confusion. It is, furthermore, apparent that there is no real reason for considering marsupials fundamentally different from the higher mammals with regard to the physiological events centering about the process of ovulation.

With the reservations suggested by the foregoing discussion, the data of Selenka, Hill, and myself must be evaluated. An inspection of text figure C discloses some very considerable discrepancies. Thus, for example, no. 314 and no. 298 yielded identical stages three and one-half and six days, respectively, postcoitum. Nos. 838 and 343 also show less

development than is to be expected on the basis of averages deduced from a study of the chart as a whole. No. 314, rather than no. 298, seems to represent the more nearly normal case and agrees well with Selenka's data as reviewed above.

It remains for us now to mention the few cases in the opossum that tend to show that ovulation stands in closer relation to the oestrous period than I at first thought, on the basis of Selenka's as well as some of my own earlier observations (Hartman, '16). The five cases are set down in the lower part of columns 1 and 2 (first and second days, text fig. C). These cases have been considered previously (Hartman, '23) in connection with the viability of the opossum ovum after its discharge.

Female no. 566 was laparotomized at 5.00 P.M. January 12, 1921, and a suspension of Ascaris eggs placed under the fimbriae. Ovulation had just taken place, for not only did the fresh hemorrhages of the discharged follicles indicate this, but (which is a better proof still), there was later found an opossum ovum which had entangled in the meshes of its albumen one of the Ascaris eggs previously placed near the abdominal orifice of the fallopian tube. In twenty-four hours the ova had reached the uterus and were somewhat degenerated; and sixteen hours later palpably more so.

No. 521. This female had ripe, bulging follicles in the ovaries on January 21, 1922, when a laparotomy was performed; forty-two hours later, eggs considerably degenerated were found in the uteri.

No. 837. This female was seen to copulate at 4.00 A.M., January 23, 1923; at 3.00 P.M. the next day, seventeen eggs, much affected by degenerative changes, were removed from the left uterus and three eggs from the tube. In this case it is highly probable that copulation had taken place too late for fertilization.

No. 762. January 25, 1923, oestrus was indicated by a suspension of cornified cells in the vaginal smear. Copulation was not observed, however. The next day the cornified cells appeared in clumps, indicating that oestrus was past. The animal was then killed. The eggs removed from the uteri had the vitellus somewhat flattened, showing that degeneration had begun.

No. 798. January 25, 1923, the vaginal examination indicated that the animal was in oestrus. Killed the next day; eggs in beginning degeneration not much different from those of the preceding.

From these cases the deduction would seem justifiable that in the opossum ovulation is pretty well bound to oestrus, though not necessarily synchronous with it.

LENGTH OF GESTATION

Hill records parturition in Dasyurus 8, 16, and 20 days after the last observed act of copulation; Selenka, 13 days less 4 hours in the opossum. Michel ('50) observed copulation January 28, 1847, and 18 days later the birth. My own case of 13 days (exact hours not recorded) agrees with that of Selenka. This author, however, for reasons detailed above, places the actual gestation at 7⅚ days. I am now convinced that 12½ days marks very nearly the actual gestation not only because of the facts already stated with reference to the postoestrous period, but also from a study of text figures C and D as a whole, that is, after taking into consideration all of the facts there presented on the rate of development. These seem to me to be sufficiently numerous to justify the conclusion that at least 13 days is necessary for the development of the embryo from copulation to birth. No embryological facts known to me seemed to necessitate raising this estimate, and all the facts seemed to argue against lowering it.

At the suggestion of Dr. H. L. Dunn, of the Department of Statistics, Johns Hopkins University, School of Hygiene, the effort was made to check the data contained in text figure C statistically. For the necessary calculations I am also indebted to Doctor Dunn.

In part I of this paper two sets of figures are cited, one on ovulation dates (203 cases), the other on parturition dates (111 cases). The figures are given in table 1. It was thought that a difference between the means of these two sets of figures ought to reflect accurately the length of the period of gestation: from mean of the ovulation days to mean of the birth days. This presumes that no other factors have entered in to warp the estimation of the raw material (table 1). It is seen from the table that for only four years (1916, 1917, 1921, 1922) is there sufficient data concerning both ovulation

and birth days. The birth dates for 1922 are somewhat bizarre; but these were used, nevertheless, for the sake of fairness. The figures are scanty, but seemed sufficient—at least the difference in the means could be judged in the light of the variations of the cases.[2] The means, standard deviations, and the probable errors of the means were determined for the two sets of material. The differences between the ovulation time and the birth time for each year were computed in days following January 1st, and the probable errors of the differences calculated from the probable error of the means by the formula:

$$\text{PE diff. of means of ovulation and birth} = \sqrt{(\text{PE ovulation})^2 + (\text{PE birth})^2}$$

The experience of the four years was then summated by adding together the four differences and averaging them and the respective probable errors. The result tells us that the average difference is 10.557 days $\pm$ 1.720 days. The period of gestation might, therefore, be anything from 8.84 days to 12.28 days. The calculated chances of the period being 12.5 days, as estimated according to text figure C, are 80 in 100. Had the data for 1922 been eliminated, the result would be considerably more favorable for the 12½-day period.

THE RATE OF CLEAVAGE DIVISION

Cleavage in the opossum egg may be considered as ending at about the thirty-two- to forty-celled stage when the blastomeres are arranged in the form of a hollow sphere (the blastocyst or vesicle) with cell margins contiguous. It is represented in text figure C as a small circle. Eleven cases from

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[2] The calculation thus made illustrates the possibilities of a statistical treatment of biological raw material, even though in this instance the material is very meager. Much more material was actually collected, but measurements were not made at the time and valuable opportunities lost. This statement is made to emphasize the need for the accumulation of accurate biometrical raw material, which will find many uses in the hands of the statisticians of the future. Perhaps more important still, the study illustrates the value of the statistical treatment of meager material in order to learn its limitations for drawing fundamental theoretical conclusions.

the two-celled to the twenty-five-celled condition have been
utilized in the chart. Since cases covering the ovulation-
cleavage stage interval were lacking, the question arose,
where in the chart the respective cleavage stages should be
placed. In other words, the starting-point of these cleavage
cases had to be determined theoretically on the basis of what
is known of other animals.

The moment of ovulation is tentatively placed at a half-day
after copulation on the basis of the rabbit, which in its de-
velopmental rate is almost identical with the opossum for the
first 10 days. In 24 hours the opossum egg reaches the
uterus in the pronuclear stage, in which condition it had
probably been for some hours. The rat reaches this stage in
24 hours postcoitum (Huber, '15); the mouse in about 11
hours (Sobotta, '95); the rabbit in 14 hours (Hensen, '76)
to 20 hours (Coste, '49), the latter being the correct time;[3]
the guinea-pig in 12 hours (Rein, '83) to 24 hours (Lams,
'12), the sheep in 1½ days (Assheton, '98). In the rat the
two-celled stage is reached in about 48 hours (Huber), in
24 hours according to Melissinos ('07). Coste, Assheton
('94), Nihoul ('26), and Rein all agree that the two-celled
stage is reached in the rabbit in one day after copulation or
14 hours after fertilization. Further examples will be given
below illustrating the agreement among authors as to the
developmental rate in this species—further evidence as to the
comparative reliability in the rabbit of estimating the ovula-
tion period from the time of first copulation. In the guinea-
pig Hensen found the two-celled stage in 24 hours; Lams, in
33 to 48 hours. In the pig Streeter and Heuser ('28) found
two-celled ova in 2 days, 3½ hours postcoitum.

Taking these comparative studies into account, together
with the fact that the opossum egg has a great deal of inert

[3] I am permitted by Mr. P. W. Gregory, Research Fellow at the Bussey Institu-
tion, to report the following results of his painstaking work on the rabbit:
Pronuclei are found up to 21 to 22 hours postcoitum; two-celled ova from 21½ to
25½ hours; four-celled ova from 26 to 31 hours. Thirty-two and one-quarter
hours is the best time to secure the third cleavage in progress; the fourth cleavage
begins around 41 hours.

yolk to expel during the first cleavage (which may or may not retard the first cleavage in comparison with the succeeding ones), I have set down the time of completion of the first cleavage at 2¼ days.

The succeeding divisions are, however, conceived to be more rapid, say ¼ day each, which is short in comparison with other forms.

For the rat Huber states that the second cleavage requires about a day (73-hour stage), which is twice as rapid as the first cleavage. The third division (89-hour stage) is still more rapid, say ⅔ of a day, the fourth cleavage occupies only ¼ day. Hence, in the rat there is clearly a speeding-up of division rates with each successive cleavage, as the blastomeres become smaller. The same might well be true of the opossum, which casts off a variable amount of yolk with cytoplasm. The rabbit ovum divides still more rapidly, for in 72 hours it has reached the morula stage and consists of at least thirty-two cells (Nihoul, van Beneden ('80), Assheton, Coste). The guinea-pig seems to be a little slower (Spee, '01), the sheep and the pig still slower. Streeter and Heuser have recently observed some cases in the pig which show roughly that the second cleavage requires about a day, the third ¾ day. But a study of nine litters from the two-celled to the sixty-four-celled stage would seem to indicate about ¾ day to a cleavage division in the pig, without any acceleration in the later divisions.

Two experiments on the opossum would have settled the time required for passage from the four-celled to the thirty-two-celled stage (three divisions) unequivocally, but for the fact that the record in one case is defective. Thus, no. 203 yielded four-celled eggs at 8.40, January 28, 1917; the second operation was done at 11.45, but whether in the morning or the night of January 29th was not put down in the protocol. The cage notes, however, pointed to the night period, which would make the interval 39 hours instead of 27. That the latter period (27 hours) is probably the correct one, however, is borne out by an entirely similar case, no. 173, in which the interval is about a day, or about ⅓ day for a single division.

Of interest in this connection are Bischoff's experiments on the rate of development in the dog, in which he removed surgically successive segments of the uterus, to note the development made in a 24-hour period in the same animal. Thus, in dog XV, the eggs passed in a day from the four-celled to the eight-celled condition; in dog XVI, from the four- to the nine- or ten-celled stage; in dog XVII, from five to seven cells to sixteen to thirty-two cells; in dog XIX, from nineteen to thirty-two or more cells.

THE EARLY VESICLE

We thus arrive in $3\frac{1}{2}$ days (no. 292, for example) at a stage when the entoderm mother cells are just beginning to proliferate. This stage corresponds roughly to the morula stage of the higher animals in which the segmentation cavity is beginning to appear. This is reached in the rabbit in about the same time as the opossum, namely, 3 days 10 hours (Coste, Assheton, Nihoul, van Beneden). In the guinea-pig, in 5 days (Spee); in the mouse, in $4\frac{1}{2}$ days (Sobotta) to 5 days (Jenkinson, '00). Six to seven days are required in the case of the sheep, according to Assheton, while the same stage is reached in the pig in 5 to 6 days, according to the same author. Streeter and Heuser find an early morula, mostly without segmentation cavity in 4 days $3\frac{1}{2}$ hours, with small segmentation cavity in 4 days 18 hours, and with well-developed cavity in 6 days $1\frac{3}{4}$ hours, and in 7 days $3\frac{1}{2}$ hours.

An examination of the chart (text fig. C) shows no. 314 to have arrived at the stage under consideration in the estimated time almost exactly; no. 173 also falls at that point. The discrepancy seen in no. 343 has already been alluded to, while the still greater discrepancy of no. 298 is to be explained on the basis of a later copulation than the observed one.

The further differentiation of the opossum blastocyst consists in a thinning out of the trophoblast and a continuance of entoderm formation at the embryonic area. This comes to have a more and more restricted proportion of the vesicle

which grows in size chiefly through the spreading out of the trophoblast. Thus litter no. 356, previously fully described (p. 63, pls. 8, 9, and 11, Hartman, '19) is placed in text figure C just in advance of the 4-day line. From the undifferentiated vesicle (nos. 193, 191) there is an easy gradation through nos. 344, 544 (fig. 1), 927, and 349 to the stage represented by no. 356. The time relations here are pure estimates based on a count of the number of cells in the ova as well as on a survey of the chart as a whole. Further experimentation is here needed.

The four-day stage (no. 356), with the exception of Rauber's layer which is characteristic of the eutherian blastocyst, corresponds very well with the 4-day rabbit vesicle as figured by Assheton, Hensen, van Beneden; with the 5½-day mouse egg (Jenkinson, Sobotta), the 6-day guinea-pig egg (Heuser, Spee, Rein), the 5- to 7-day pig (Assheton, Corner, Streeter and Heuser), the 9- to 10-day sheep (Assheton). The rabbit and the opossum are, therefore, in advance of other mammals so far as known on the fourth day of development.

THE BILAMINAR BLASTOCYST

The next landmark which we may select is the bilaminar blastocyst between 1.0 and 1.5 mm. in diameter, fully described by Selenka, and by Hartman ('16, '19). In both the rabbit and the opossum the vesicle at this stage is practically spherical (figs. 5, 9, 21), in the dog elliptical; in the rat, the mouse, and the guinea-pig it is more or less collapsed, while in the pig and the sheep it is beginning greatly to elongate. Bischoff found in the dog that 25½ hours were insufficient for the egg to pass through the bilaminar stage, vesicles of 2.0 × 2.5 mm. having in that interval grown to 3.5 × 4.5 mm., all still didermic. In the opossum the growth of the vesicle is at the expense of the albumen layer—a feature shown by the sketches in the charts (text figs. C and D). With the growth of the vesicle in diameter the entoderm spreads toward the lower pole of the egg. Thus in ova no. 352 (text fig. D and Hartman, '19) and 931 (text fig. C) the entoderm has not

yet reached the equator. Other batches of about this stage of advancement are nos. 931, 194, and Selenka's 10-hour' stage (actually 5 days 10 hours). It will be noted that copulation ages are known for both no. 194 and Selenka's case; no. 931 falls naturally on the 5-day line in comparison with other litters of the chart. Hence in text figure D, no. 352 is placed on the 5-day line to mark the beginning of the chain of stages there shown. This chart contains a sufficient number of cases to be a fairly reliable picture of what happens from the end of the 5th day to the completion of 8½ days.

The bilaminar stage is completed, that is, the entoderm has reached the lower pole of the egg in nos. 205, 352, and 347. A day and a half later (no. 353, figs. 22 and 23) the primitive-streak mesoderm begins to proliferate; hence it is apparent that the bilaminar stage in the opossum, as in the dog, persists for over a day. It marks the half-way stage in the actual period of gestation, and yet there is no embryo as yet, only what MacDowell, Allen, and MacDowell ('27) call the 'proembryo.'

Studying text fig. C as a whole, 6½ days may be set down as a fair average for the 1-mm. bilaminar blastocyst, which is exactly Selenka's finding as corrected (v.s.). Our 191 and 293, 285 and 347 accord with this estimate, while nos. 838, 343, and 306 fall 12 to 24 hours later. The first two have copulation data; hence it is possible that our estimate of 6½ days may need some revision in the future. One-half day later, the primitive-streak stage is well indicated.

THE PRIMITIVE-STREAK STAGE

This rather clear-cut stage has been described in a number of mammals, with copulation age in a few of them. In the rabbit Minot and Taylor ('05) find it 6½ days postcoitum. However, this seems an exceptionally early estimate, in view of the findings of other authors, as well as in consideration of the fact that Minot and Taylor themselves find but slightly more advanced stages 7½ and 8½ days postcoitum. Hence, we may accept Hensen's 7-day term as more nearly the modal

one. Sobotta thought the mouse reached this stage in 8 days
or more, while MacDowell, Allen, and MacDowell find 7½
days sufficient for the establishment of a primitive groove
and the beginning of a head process. They state further:
"Of twenty-one 7-day embryos from three litters, sectioned
transversely or longitudinally, sixteen are sufficiently devel-
oped for a primitive groove, but none show a head fold."
The mouse is thus almost exactly at the same stage as the
opossum at 7 and 7½ days. The guinea-pig appears to speed
up at this point, if we are to accept Bischoff's time of 6 days
14 hours and Kölliker's 6 days 18 hours. But Kölliker also
reports a bilaminar blastocyst at 7 days. These data are
clearly at variance with Hensen's egg cylinders that have
not yet any mesoderm at 11 days and Lieberkühn's areas with
primitive streak and considerable mesoderm at 13 days and
13 days 20 hours. Hensen's data on rate of development be-
tween the inner cell mass stage of 6 days and the six-somite
stage at 14 days likewise need checking up by future work.
In the case of the dog the primitive-streak stage is reached
in 16 days, according to Bonnet; in the sheep in about 14½
days, according to both Keibel and Bonnet.

Of our own batches of eggs nos. 380 (figs. 15 and 16), 486
(fig. 17), and 578 are found to be in the same stage of devel-
opment, although for the last-mentioned there are only a
photograph of the eggs in salt solution and a verbal brief
description extant; hence the eggs could not be reexamined
for details, and their exact position in the chart is slightly
problematical. The cue for the proper placing of these primi-
tive-groove stages in text figure D is furnished by batch no.
211, in which the interval of 12½ hours intervened between
an early primitive-streak stage and one having a short medul-
lary groove with a spread of mesoderm somewhat beyond the
limits of the embryonic shield. Nos. 377 and 284 are some-
what in advance of no. 211. No. 380', with its single pair of
somites just beginning (fig. 19) is still more advanced,
though about the same as no. 349 of text figure C, which falls
on the 7½-day line. No. 356' at 7⅓ days and no. 298' at

9½ days (fig. 29) are midway between 377 and 380′, their medullary grooves being about the same length as the primitive streaks and the notochord or head process half as long. No. 298′ is, as already indicated, palpably out of place in the chart (text fig. C), as is the earlier stage secured from the same animal. The corresponding stages represented by Selenka (line 2 of text fig. C) are seen to be approximately in agreement with my own chart; the slight inconsistencies are, moreover, readily explained by the fact that the various stages came in part from different animals (v.s.).

For the dog, Bischoff records some further interesting experiments that are of unusual interest in connection with the stages shown in the latter part of text figure D. In his specimen XLV he removed a primitive-streak embryo corresponding to our no. 380 (fig. 15) or no. 486 (fig. 17) and 24 hours later a stage quite comparable to our nos. 298′ (fig. 29) and 356′, a rate of differentiation about half as rapid as in the opossum. Again, his no. XLVI yielded about the same stage as our no. 377, and in 24 hours a stage attained by no. 377′ in 12 hours. This interesting comparison perhaps indicates a speeding-up in differentiation rather than growth in the opossum as compared with an animal whose birth is still far removed in time. There is need of more extensive work along this line in mammals, including the opossum. Certainly, such studies should carefully distinguish between growth in size and differentiation of parts. Counting mitotic figures in an embryo is an index of rate of growth, but not of differentiation.

THE EMBRYOS

Embryos with six to ten somites are to be expected in the opossum about 8½ days postcoitum. At this stage the brain vesicles may be made out and the pericardial cavity has the typical horseshoe shape about the head end of the embryo. The latter is, however, to be seen as early as the stage represented by no. 356 (Hartman, '19). One animal (no. 340) furnished two sets of embryos at about seven to ten somites

6¼ hours apart—an interval of time quite sufficient to add
to the complexity of the brain, the number of somites, and
the head fold.

With the 8½-day opossum embryo the rabbit again agrees
very well. Thus Minot and Taylor found a two-somite rabbit
embryo at 8¼ days, one of seven somites at 8½ days. Hensen
found a three-somite embryo in 8⅓ days, one of more somites
at 8½ days. Double this time is required in the pig (Keibel)
and the sheep (Bonnet).

The older embryos are represented in text figure C by out-
lines that admit of the respective stages being made out with
reference to the head and the limbs. In plates 1, 2, and 3
seven embryos are shown photographically. In the head the
gill arches are indicated in the younger embryos (fig. 14);
such a stage may be expected in both the rabbit and the opos-
sum at 10 days. In the later embryos the optic pits may be
seen. The mouth is at first wide open, almost closes later,
and near term comes to be surrounded by the peculiar muzzle
(Schnabelschild), as well described by Selenka. The fore
limb is precocious, for near term the fingers are provided
with claws adapted for crawling into the pouch (Hartman,
'20) while the hind limbs are still embryonic paddles.

SUMMARY

In constructing our charts (text figs. C and D), we have
assumed thirteen days for the entire period of gestation, from
coitus to parturition, and have limited the outline of the chart
with straight lines, as though the span were always invari-
ably the same. This was done as a matter of convenience.
There can be no doubt that the span of gestation is subject to
enormous variations, as is well known for all mammalian
animals and the human species as well. The opossum can
be no exception. One might even expect a greater percentage
of variation in the opossum because of the early birth, since
the physiological processes that cause parturition at the ap-
propriate time (still unknown) must be delicately adjusted
and might be thought of as rather poorly established in the

presence of corpora lutea only twelve and one-half days old. Indeed, several cases among my records of two- to four-day intervals toward the end of gestation point rather significantly to an occasional period longer than thirteen days. Statistically, as shown above, the chances are against the assumption of a longer average period of thirteen days between copulation and parturition and twelve and one-half days between fertilization and birth.

It is apparent, moreover, that the first half of gestation is entirely concerned with the differentiation of the 'pro-embryo'; hence the actual intra-uterine development of the embryo itself comprises but five and one-half days, for at seven and one-half days the primitive streak is completed and the medullary plate begins to differentiate; the notochord to grow forward from Hensen's node. As one views text figure C, one must truly marvel at the complexity of even the visible changes which occur in the period represented by the latter half of the chart.

III. THE POSTNATAL GROWTH AND DEVELOPMENT

Though not as extensive as might be desired, the data upon which the postnatal development is based afford results which should prove of interest and value. For the opossum these new data stand alone, except for the four or five cases cited by Barton (1823), Meigs ('47), Michel ('50), and Audubon and Bachmann ('51). Of these the data given by the last on sizes at various ages are clearly wrong, unless I mistake the use of the grain as a unit of measure, which I take to be 0.0648 gram.

For the convenience of the reader, the new data have been reduced to the form of charts and growth curves, which may serve as a basis for discussion. The interested reader may refer for details to the appendices.

Before proceeding with the subject of growth, it is necessary to present some relations of body length to weight; for this information has been in part utilized in translating length to weight for text figure G and weight to length for text figure H in a few cases.

DATA OF LENGTH ON WEIGHT

The weights of the animals in grams is accurate, the measurements of length as accurate as the method employed. It was found best to use the snout-rump dimension, rather than crown-rump, for the latter depends upon the amount of curvature allowed the neck region. To secure the measurement, the dead animal, if large, is laid on its back, a rule is laid along the ventral surface, and the length from the tip of the snout to the junction of the tail with the trunk read off. A
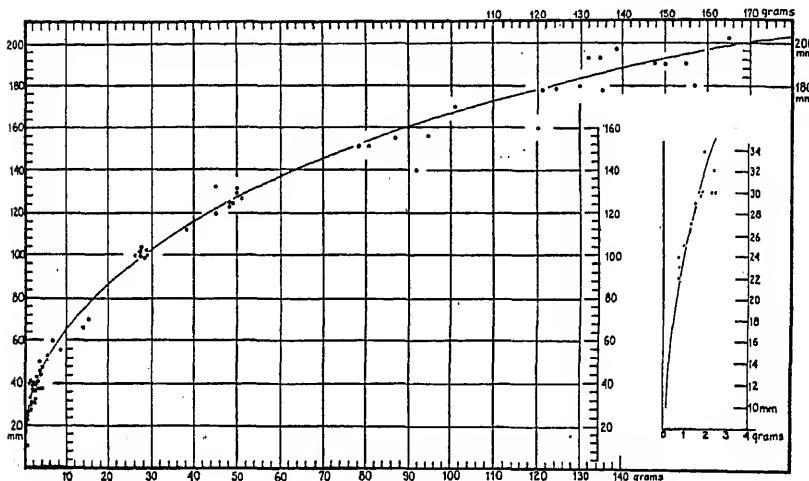
Text fig. E Curve of length in millimeters on weight in grams for sixty-eight pouch young of the opossum, both sexes.

small pouch young is held extended with the fingers of one hand and a rule applied to the ventral surface.

Some sixty-nine young animals of both sexes, still under the protecting care of the mother, if not actually sucking, were both measured and weighed. These range from about 10 mm. in length and a little over 0.1 gram in weight to about 200 mm. in length, 165 grams in weight. The plotted curve shown in text figure E has the usual shape of length-weight curves, as, for example, Streeter's curve of human material ('20).

Of adult or adolescent animals, females only were weighed and measured, a total of 115. An additional seventy-seven furnished weights only. The former were used to construct the curve in text figure F, to which has been added a portion of text figure E to complete the first part of the curve.
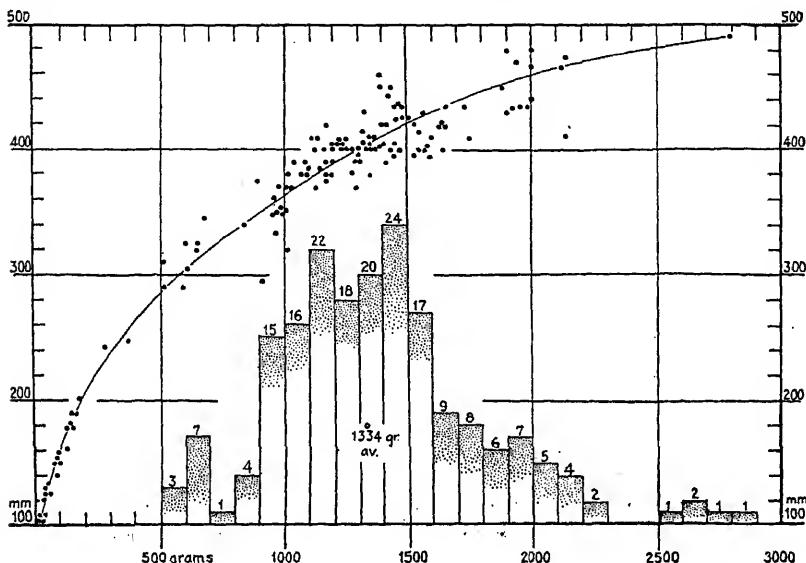
Text fig. F   Curve of length in millimeters on weight in grams for 117 mature female opossums. At the beginning of the curve are added some prepuberty cases (under 200 mm.) from text figure E. Below the curve, graph of 192 female opossums arranged by weight and grouped according to 100-gram columns, from 5 to 600 grams up. 1334 grams, the 'center of gravity' of the graph, represents the 'average' female opossum capable of bearing young.

From text figure F it appears that somewhat over half of the females range from 1000 to 1500 grams (2.2 to 3.3 pounds) and measure 375 to 420 mm., and that this group is pretty evenly distributed about the point of intersection of the 400-mm. and the 1250-gram line. An average animal of such size I find is frequently designated in the protocols as 'medium' in size. Only one female weighed as much as 2800 grams (no. 429), the largest female ever weighed by us, though I have the impression that several were encountered

that struck one as slightly larger. Audubon and Bachmann
must certainly have been mistaken when they described a full-
grown female measuring 15½ inches (394 mm.) as weighing
12 pounds.

The curve indicates, furthermore, that the opossum, like
other animals, the rat, for example (Donaldson, '24), begins
breeding long before she is full grown. The 115 dots in the
chart represent not fully grown animals only, for many
among them were seen to be palpably young; others are pal-
pably old, of course, having permanently dilated pouches and
enlarged nipple bases. The animals are simply random sam-
ples of females, mostly pregnant or at least approaching
closely to their first oestrus, which happened to be unfor-
tunate enough to be brought into the laboratory.

Of pregnant or pseudopregnant, and therefore sexually
mature and breeding females, the following represent ex-
tremes in size.

No. 226, 660 grams, 345 mm.; had 10.5-mm. embryos (near term)
February 11, 1915.

No. 234, 650 grams, 350 mm.; was pseudopregnant March 17, 1915.
But this female was a black one and may have belonged to the
species D. marsupialis; hence the preceding may be taken as the
smallest breeding specimen of D. virginianus among my records.

No. 429 was the largest female, 2800 grams, 490 mm. She had
pouch young about two weeks old, February 13, 1927.

No. 702, the next largest female, weighed 2720 grams, February 7,
1922, when young, unfertilized eggs were removed from the uterus.

Finally, attention may be called to certain descriptive
terms for size applied to specimens whose records were being
set down, such as 'tiny,' 'small,' 'medium,' 'large,' 'very
large.' These terms were used along with other descriptive
features, to make more certain the identification of experi-
mental animals whose number otherwise was read off from
a code of holes, slits, and notches in the ears in connection
with cut-off stubs of vibrissae. Sometimes the animal was
weighed or measured later. Hence, it was possible, by col-
lating these cases, to define the general terms for size more
precisely. The result is as follows:

Descriptive adjective	Weight (grams)	Length (mm.)
Tiny	1000 or less	355 or less
Small	900 – 1300	350 – 390
Medium	1200 – 1400	375 – 410
Large	1300 – 2200	395 – 440
Very large	2000 or more	430 or more

THE GROWTH CURVES

Two charts are presented, one of weight on age (text fig. G), and the other snout-rump length on age (text fig. H). The former covers fifty-six days, the latter, thanks to data furnished by Doctor Langworthy, almost ninety days. Doctor Langworthy's cases are represented in text figure H by open rings, my own cases, as usual, by dots. Since Doctor Langworthy's cases were not weighed, those that come within the scope of the weight-age chart were translated into terms of weight with the aid of text figure E and entered in text figure G with open rings.

THE NEWBORN

The newborn pouch young have been variousy measured and weighed. Barton states that the smallest specimens of one litter weighed barely 1 grain (0.0648 gram); another specimen barely 2 grains (0.13 gram) and the remaining five, taken together, exactly 7 grains—an average of about 0.9 gram. Schwarz ('20) found sixteen young in a pouch; these filled a tablespoon and weighed 2 grains (0.13 gram) each. Meigs was not certain of the exact day of birth of his litter, one of which he found to weigh 3½ grains (0.227 gram), which seems about right for a pouch young two days old. Audubon and Bachmann's birth weight of the opossum is still at variance with the other figures quoted and those of Meigs. These authors state that one embryo recovered by caesarean section weighed 4 grains, or 0.26 gram; others weighed 3½ to 3¾ grains at birth. Incidentally, it may be stated that these authors' estimate of the seven-day pouch young at 30 grains (about 2 grams) and the ten-day pouch young at 22 grains (1.43 grams) are also rather far removed from my own curve; their cases are entered in text figure G at x and x'.

Selenka failed to weigh the newborn that came into his possession, but he measured one which he found to be 13 mm. in length. I also failed to weigh in the fresh state the young whose birth I witnessed in 1918 (Hartman, '18); but after storage for nine years in 80 per cent alcohol after fixation in Bouin's fluid the eleven remaining specimens out of the litter of eighteen weigh as follows: 0.125, 0.116, 0.112, 0.110, 0.108, 0.107, 0.105, 0.103, 0.1, 0.95, 0.09 gram. Dehydration causes shrinkage, of course; but it is probable that in life the embryos weighed in the neighborhood of 0.13 gram. Another female, stored in a shallow table drawer about 6 inches deep, overnight, was found the next morning to have given birth to ten young, of which only one was attached. This one weighed 0.16 gram; its stomach was filled with milk. The nine others that had perished weighed a total of 1.2 grams—an average of 0.133 gram.

In view of the aforementioned facts, it is safe to place the average weight of a newborn pouch young at 0.13 gram. If we assume that ten is an average litter born, the ratio of body weight of the litter to the mother is about 1:1000. Perhaps the bear is a close second to marsupials in the disparity between the size of the mother and the offspring at birth. This was first called to my attention by Dr. Francis G. Benedict, of the Carnegie Nutrition Laboratory, Boston, who presented me with the photographs of a pair of cubs measuring 210 mm. in snout-rump length and weighing about a half-pound each. The ratio in this case would be one to four or five hundred. Brehm states in his 'Tierleben' that the polar-bear cubs weigh 750 grams each, or about 3 pounds for the pair. If we take the maximum weight of a polar-bear female at 1500 pounds, we get the ratio of 1:500. Probably the other extreme in the mammalian series is found in the bat, where the single fetus may weigh fully one-third as much as the mother (!)[4]

[4] I am indebted to Dr. C. Hart Merriam for information (private communication) which indicates that in bears the ratio of the weight of the cubs to that of the mother is considerably greater than 1:500. Thus a Yellowstone Park

The further growth in weight is plotted in text figure G; in length, in text figure H. In the latter, Barton's sixty-day young, weighing 35 grams, is entered at x and Meigs' seventy-four-day young, weighing 27.8 grams, is entered at x', after
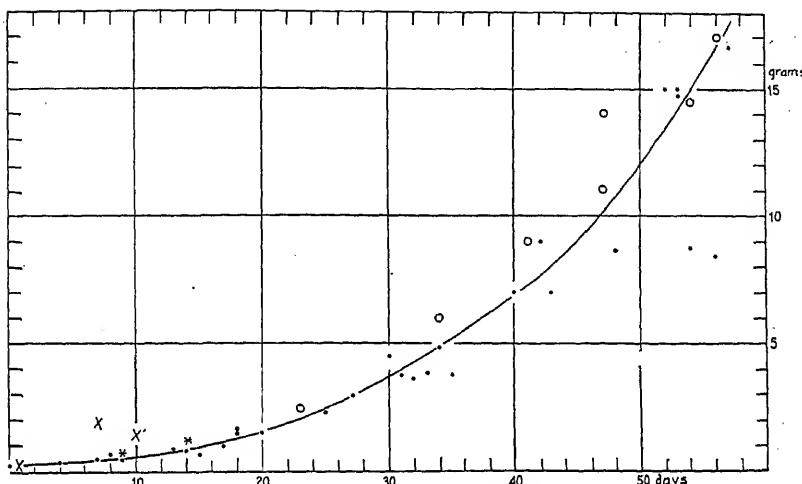
Text fig. G. Growth curve of the opossum pouch young, weight on age, for the first fifty-six days. Each dot represents the average of a litter (details given in appendix C). xx, Audubon and Bachmann's data, apparently wrong. **, data by Meigs, nine days, fourteen days (corrected).

these weights were reduced to length equivalents (107 and 100 mm., respectively) by the aid of text figure E. No other data on rate of postnatal development in the opossum are known to me.

It will be seen that the growth curve thus far closely parallels the first part of the growth curve of other mammals and

grizzly in the National Zoological Park at Washington, D. C., gave birth to two or three cubs (number uncertain), January 13, 1913. One cub died at five days of age; it weighed 9½ ounces. The mother weighed 390 pounds the year before. A Yellowstone Park black bear at the same Zoo. lost one of its two cubs the day after its birth, when it weighed 9 ounces (Baker, Smithsonian Miscl. Coll., vol. 45, p. 178, January 7, 1904). A European brown bear, Ursus arctus, at the Philadelphia Zoological Park, thirty-six hours after its birth, weighed 12¾ ounces (Forest and Stream, p. 84, Feb. 4, 1899).

man.[5] In other words, the opossum is still long after birth, to all intents and purposes, an embryo, although leading, as Doctor Meigs has grandiloquently put it, "a chylopoietic, warm-blooded, oxygenating, innervating, and free-willing life."
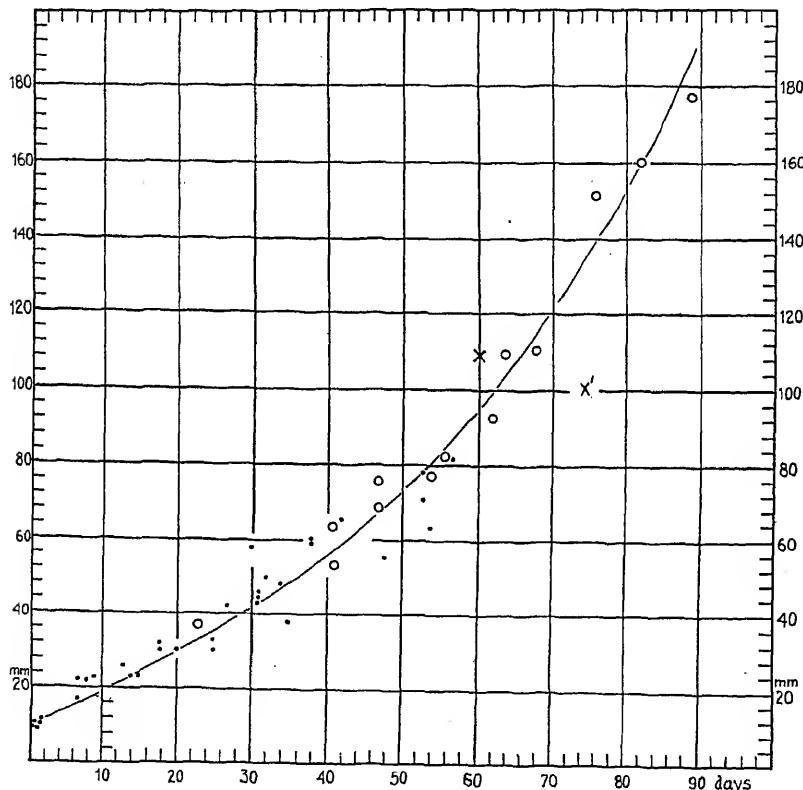
Text fig. H Growth curve of the opossum pouch young, length on age, for the first ninety days. Dots represent averages of litters. Circles are individuals measured by Doctor Langworthy, who kindly turned the data over to the writer. x', Meigs' case: seventy-six days, 100 mm., calculated length equivalent; x, Barton's case: sixty days, 107 mm., calculated length equivalent.

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[5] Chapters 1 and 2 of Pearl's "The biology of population growth" gives a good summary of "How things grow." It contains numerous normal growth curves for various plants, animals, and populations.

THE FIRST APPEARANCE OF HAIR

The opossum young are born naked as tiny red flesh-like masses. In several days the color changes to a healthy pink, or typical flesh color. My earliest record of 'hairy' young concerns the pouch young of no. 116, forty-two days old, weighing 9.0 grams and measuring 65 mm. However, examining the alcoholic specimens of litter no. 593 a, less than 5.0 grams in weight, thirty-four days old, I find that these are already provided with down easily visible under the hand lens. The hair is barely visible in the pouch young of no. 861, thirty-one days old. The 15-gram pouch young of no. 883, fifty-three days old, have hair that is distinctly pigmented with the color characteristic of the species and the color phase of the animal.

PERIOD OF ATTACHMENT TO THE TEAT

Barton states that the young are continuously attached to the teat for fifty to fifty-two days, when they are about the size of a common house mouse. In the case of the female kept under observation by Meigs, the young remained 'undetached' for seventy-four days (two days are added to the figure given by the author); but two days later, one embryo, with eyes slightly open, was crawling on the body of its dam. Audubon and Bachmann state that in four weeks the young relax their hold and peep out of the pouch; in five weeks they crawl on their mother's back. It is needless to add that here again these naturalists are mistaken in their estimates of age.

Between the estimates of the last-named authors and Meigs there is a 100 per cent discrepancy; between the observations of Meigs and Barton, 40 per cent. The latter I regard within the limits of normal variability, if I may judge, for example, from my own data on the time the eyes open. In certain crucial cases, no. 905, 907, and 1007, the notes fail to state whether the young were attached or not. However, the young of no. 876, fifty-seven days old, weighing 12.5 to 18.3 grams, were still attached; the young of no. 150, about the size of mice (25 grams, 95 mm.), were running freely about on the mother.

In general it may be said, then, that the young begin to leave the teat for the first time when about the size of a house mouse and between fifty-two and seventy-four days old. This relates to animals kept in captivity.

The question is sometimes asked whether the pouch young, if removed from the teat by the experimenter before the time intended by nature, can find their way back and get hold again. This must be answered in the affirmative. Audubon and Bachmann tested this out by experiment and found that not only a mother's own young, but even the larger young of another female would attach themselves anew. I removed newly born young and found they could reattach themselves; indeed, one young found the teat three successive times. On one occasion the two large, hairy young of one female were found missing; one was crawling about in the cage, the other had found a foster mother. The free individual was placed on its mother teat, but would not take hold while held by the observer's hand, hence it was dropped into the pouch. An hour later, it was found attached to the teat.

OPENING OF EYES AND MOUTH

The mouth of the embryo is at first a wide cleft which gradually closes toward the end of gestation until only a circular orifice is left for the reception of the teat. Later the lips form and separate like the eyelids and about the same time. But there is great variation in this respect, as suggested above. An inspection of the data presented in Appendix C will make this clear. Thus, for example, litter no. 883, weighing about 15 grams, is made up of individuals with eyes open, closed, or one eye open, the other closed. The individuals of litter no. 1007 have the eyes still closed, although they were over 27 grams in weight—nearly double that of the preceding whose eyes were in part open. Within a single litter there may be great discrepancies in both size and differentiation, as, for example, in litter no. 876, in which the smallest member, weighing 50 per cent less than its fellows, had its eyes and mouth open, whereas the largest member had its eyes still closed, its mouth open on one side only.

WEANING AND INDEPENDENCE

After leaving the teat, the pouch young does not yet leave the mother, but clings to her fur and makes periodic visits to the mammae for nursing. At the approach of danger the young scamper into the pouch, wherein the mother encloses them with the sphincter as well as possible, in consideration of the size of the litter and the individuals comprising it. Lactating young are sunned at times by the mother and may be seen, as I have, crawling about the mother while she lies curled up in sleep. Doctor Barton stated that even after the mother gives birth to a new litter she "does not withdraw from them (the older litter) her useful attention and assistance." Audubon and Bachmann believed that the young are only two months with the mother, but continue in the vicinity till autumn. "In the meantime," they say, "a second and often a third brood is produced and thus two or more broods of different ages may be seen, sometimes with the mother, and at other times not far off." Parenthetically, attention may again be called to these authors' low estimate of the rate of development in the opossum. They give no specific cases to warrant the exaggerated statement quoted above. They cite an interesting case, however, of a female that had in her pouch five very small young, while seven others, about the size of full-grown rats, were detected peeping from under rubbish. Other similar cases might be cited as follows:

Barton secured on May 14th a female that had five young as large as a two-thirds-grown rat; seven days later, she gave birth to a new litter of seven young. I doubt not that the older litter had been weaned some time before the birth of the second litter. This case is paralleled by my own no. 1001, which on May 1, 1925, was encumbered with a litter of six young the size of rats (140 to 155 grams in weight), while in the pouch was a newly born young and signs of the former presence of others. The older litter had been weaned some time before, for the teats had greatly retrogressed and returned to a size consistent with the reception of the new litter. In this connection the specimen reported by Meigs is

of interest. In 1833, he captured at Camden, New Jersey, a female with five young, each as large as a half-grown rat and still unweaned. He saw them "take the teat, and creep on the mother's back and muzzle, and hold on by means of the prehensile tail, wrapped round her ear, or round her leg."

From the above-cited cases it seems clear that weaning takes place a considerable time after the young attain the size of mice, and some time after they reach the size of a half-grown rat. I should add that this happens when the young are at least two and one-half months old.

Another case should be mentioned in this connection, that of female no. 150 (Appendix C). April 17, 1915, she had young about 95 mm. long, running freely about her. Thirty-eight days later, the young had attained the size of rats; their estimated age at this time was one hundred days. It is probable that in a state of nature these young would have begun an independent existence. Certainly, the young of no. 939, measuring 250 mm. in snout-rump length (about 350 grams in weight), had been independent for a week when killed. Such litters keep together ('den-up' together, as hunters would say) for some time, as Audubon and Bachmann described them. The records of the Biological Survey contain a number of just such references. A photograph of such a young opossum may be seen on page 276 of the National Geographical Magazine, vol. 52, no. 3, September, 1927. According to the legend, there were five of these young opossums together, the other four having escaped, which renders it likely that in this case also the litter was held together as a family by the mother.

SUMMARY

The newborn opossum weighs about 0.13 gram. An average litter might weigh one one-thousandth of the body weight of the mother. The growth curve for weight is given for fifty-six days, for length for ninety days, and is, for the period covered, the shape of an embryonic growth curve of higher mammals and man.

The eyes and mouth open as early as the fiftieth day, when the pouch young are as large as small mice or may remain closed considerably longer—until the size of a large house mouse. They are weaned when about eighty days of age. Soon thereafter the mother may again become pregnant with a second litter, but the weaned young still remain with the mother for some time. It probably takes at least ninety days to raise a litter of young to a state of independence.

DISCUSSION

In studies of the kind here undertaken one is struck by the extent of the variations in rate of development involved, which are, of course, only expressions of the heterozygosity of germ cells. Rate of development differs from one race to another and, since individuals of no large litter are ever exactly alike, the rate differs from one individual to another. Note, for example, the variation within litters of pouch young as given in the second part of this paper. Hence, data on rate of intra-uterine development will never be quite 'satisfactory' in the sense of being exactly predictable, or enabling the collector to secure with certainty the precise stage that he may desire. The greatest source of error, however, in making a Normen-tafel for the mammal lies in the difficulty thus far experienced in determining the exact hour of conception or fertilization of the ovum.

As pointed out, embryos are usually dated from the time of copulation. But the heat period may last days or even weeks; so that between the first and the last copulation as many as seventeen days have been recorded (Bischoff, '45). What a percentage of difference this may make in calculating the age of a two-day embryo! But this percentage may be very high in the case of the younger stages and the physiological events that center around ovulation, even in those animals that exhibit brief heat periods. The method has proved so elusive that numerous authors have timed their specimens from the hour of parturition in forms (the laboratory rodents) in which ovulation is known to follow close onto

parturition. But the relation of heat or oestrus to ovulation is itself not fixed and is subject to variations common to all physiological processes, and these time relations vary between large limits.

In this connection I may hazard three suggestions as to how this problem may in part be met. In the first place, the species used for the experimental work should be favorable. If it is selected on the basis of availability and the anatomy and physiology of the reproductive organs, the rabbit, rat, mouse, guinea-pig, and opossum are the only species worthy of consideration at present. The rabbit has the advantage of ovulating almost exactly ten hours after copulation, as has been known since Coste began his excellent work in 1834; and this explains the agreement among workers on this species. The rabbit differs from most mammals in that the female will accept the male and is able to ovulate over long periods, for the ripe graafian follicles appear to remain quiescent and 'ready' for a very considerable portion of the oestrous cycle. The rat, the mouse, and the guinea-pig, as well as the opossum, have definite oestrous periods easily studied by Stockard and Papanicolaou's vaginal-smear method now in universal use; by this method the events of the cycle, including ovulation, may be approximately determined and the next step greatly facilitated. These rodents, furthermore, ovulate and accept the male soon after parturition.

The second step suggested consists of verification by laparotomy; if necessary, by successive laparotomies. These need not seriously affect the reproductive capacity of the animal (Hartman, '19; MacDowell, '27; Corner, '27). And finally we must return to the method of Bischoff, described in part II of this paper, namely, of surgically removing a uterus or portions of the uterine horns and noting the progressive development in the surviving egg chambers. Furthermore, in any mammal two cleavage stages might be secured, with accurate intervals, by successive removal of the fallopian tubes. It is believed that such material, timed to the minute, is more valuable than the same quantity or more

material based on copulation ages of different animals. It is this method that has furnished the greater part of the data on which the intra-uterine development of the opossum, as above set forth, is based.

Finally, it has been shown that intensive experimentation within closely defined limits is needed on the early ontogeny of mammals, such, for example, like the recently published study on the growth curve of the mouse by MacDowell, Allen, and MacDowell.

SUMMARY

In the first part of this paper the optimum time for collecting pregnant opossums at Austin, Texas, is discussed. The breeding season begins in January after a three months' anoestrous period, and in the third and fourth weeks the modal point for the number of conceptions is reached. After this period, most of the animals captured in the field are pregnant or have pouch young.

The rate of intra-uterine development (part II) was investigated chiefly by the method of surgical removal of one uterus, noting the stage of eggs or embryos which it contains, and allowing the surviving uterus to incubate its ova a precalculated period of time. Unique charts (text figs. C and D) allow the reader to see the development of different intervals at a glance. Thirteen days is probably near the truth for the average period from copulation to birth; twelve and one-half days for the actual prenatal development. Comparison is made with other mammals concerning the time when certain well-defined stages are reached—information which has been brought together here probably for the first time. It is shown that the rate of development of the opossum for the first ten days, at least, is most like that of the rabbit, representative of one of the most primitive orders of Eutheria. The primitive-streak stage is reached in seven and one-half days, which leaves but five and one-half days for the actual development of the embryo itself to the time of birth.

The curve of postnatal development has the shape of the
embryonic growth curve of the higher mammals, for birth is
so early in ontogeny that it does not constitute the momen-
tous physiological event as in the higher mammals.   The
pouch young begin to open their eyes and lips when about
fifty days old and when they have attained the size of a full-
grown house mouse.   At this time they let go of the nipples
for the first time, but continue to nurse for thirty days more.
Even then they take advantage of the protecting care of the
mother until they attain the size of the full-grown Norwegian
rat, when they are ready to shift for themselves.   The mother
again becomes pregnant soon after weaning the first brood.
It therefore requires quite all of three months to rear one
brood of young.   It is doubtful if a given female can rear more
than two broods a year, except in rare instances, since the
last three months of the calendar year constitute the
anoestrum.

APPENDIX A

Extracts from the protocols of animals furnishing data for the
construction of charts 1 and 2 (rate of intra-uterine development)
and not mentioned in my paper of 1919 (Jour. Morph., vol. 32,
pp. 8–16).   It was deemed unnecessary here to repeat the protocols
previously published.   The new material here presented was col-
lected since 1919 or concerns only stages from the primitive-streak
stage on.   ('Chart 1' = text figure C; 'chart 2' = text figure D.)

No. 114.   Copulation observed 10.50 to 11.00 A.M., February 6, 1915.   February
11th, 2.30 P.M., a laparotomy was performed; no eggs were found.   February 15th,
10.00 A.M., 11-somite embryos (chart 1).

No. 134.   Two sets of embryos, sixteen hours apart, were removed from this
female, the first litter nine days after copulation.   A representative of the second
litter is seen in figure 20.

No. 207.   January 29, 1916, five vesicles about 4.2 mm. in diameter and five
unfertilized eggs.   Sixty hours later, three vesicles, 11 mm. in diameter, and
seven dead vesicles or unfertilized eggs.   The normal vesicles contain 4-mm.
embryos, according to the notes.   These specimens are not entered in the charts.

No. 211   (Didelphis marsupialis from San Benito, Texas).   February 2, 1916,
thirteen blastocysts in early primitive-streak stage, no unfertilized eggs.   Measure-
ments are as follows:

| In salt solution | 2.5 | 2.5 | 2.36 | 1.78 | 2.3 | 2.0 | 2.6 | 2.3 | 2.26 | 2.24 | 2.13 |
| In fixing fluid | 2.8 | 2.6 | 2.50 | 1.75 | 1.95 | 1.86 | 2.45 | 2.3 | 2.28 | 2.15 | 2.05 |

Twelve and one-half hours later, vesicles with pear-shaped embryonic areas and short medullary grooves. They measure as follows (chart 2):

| In salt solution | 3.6 | 3.52 | 3.52 | 3.46 | 3.37 | 3.37 | 3.26 | 2.8 |
| In fixing fluid | 3.3 | 3.2 | 3.16 | 3.16 | 3.06 | 3.06 | 3.02 | 2.65 |

No. 284. January 13, 1917, four vesicles, measuring 4.5 to 4.7 mm. Fourteen and one-half hours later, ten vesicles, measuring 9.5, 9.0, 9.0, 8.9, 8.7, 8.7, 8.7, 5.5, 4.0 mm. The former set are comparable to 298′, 344, and 356′; the latter contain embryos with 8 to 10 somites. These specimens are not entered in the charts.

No. 298. Figure 29 is introduced to show the stage of advancement reached three and one-half days after the 60 to 120-celled blastodermic vesicle (Hartman, '19, p. 13, for protocol). (Chart 1.)

No. 314. Figure 13 shows an embryo about nine days after an observed copulation (chart 1).

No. 334. January 27, 1917, eleven vesicles, 2.75 to 3.0 mm. in diameter, slightly elliptical embryonic areas, late primitive-streak stage, no medullary groove, mesoderm has reached the anterior margin of embryonic area. Twenty hours later, one defective 5.0-mm. vesicle with embryo of 6 somites and two unfertilized eggs. The vesicle is small in proportion to the differentiation of the embryo (chart 2).

No. 337. Figure 26 shows one of the larger eggs photographed fresh in utero and shown in figure 10, Hartman ('19). The mesoderm has almost reached the anterior margin of the formative area (the light streak is free of mesoderm). This represents four and one-half days' development after the sixteen-celled stage (chart 1).

No. 340. Six and one-quarter hours' development represents, in two batches of vesicles from this female, an increase of about 2 somites (twelve to fourteen), and an easily recognizable increase in differentiation of brain contour; the otic vesicles also appeared in the interval. These specimens are not entered in the charts.

No. 344. Figure 28 is introduced to show the spread of the mesoderm, the primitive streak, and short medullary groove, a stage reached three and one-sixth days after the early proliferation of entoderm, as detailed in Hartman ('19, p. 15) (chart 1).

No. 346. See figures 21 and 24; also Hartman ('19, p. 15).

No. 349. The notes given for this specimen, Hartman ('19, p. 16), need correction in so far as the vesicles at the second operation (three and one-third days after the first) measured 8 mm. The eggs are no longer extant for examination. The embryos had only a few somites (chart 1).

No. 353. See figures 22 and 23; also Hartman ('19, p. 16).

No. 356. January 30, 1917, young vesicles almost identical with those of no. 544 (fig. 1) and fully described in Hartman ('19). Three days three and three-quarter hours later, the ova measure 4.9, 4.6, 4.25, 4.4, 3.25, 1.5 mm. (the last two moribund). The stage is almost identical with no. 298′, shown in figure 29.

No. 360. January 30, 1917, bilaminar blastocysts, about 1.5 mm. in diameter (Hartman, '19, pl. 8). Three days later, 5.75-mm. embryos like that shown in figure 14. This interval has been added to that of no. 306 of chart 1, for no. 306′ is almost identical with no. 360.

No. 377. February 1, 1917, the 5- to 6-mm. vesicles; medullary plate only a little less developed than in no. 356' and 298'. Thirteen hours later, the embryos have about 10 somites and brain with optic pits (chart 2).

No. 380. February 3, 1917. 3.0- to 3.5-mm. vesicles with pear-shaped formative areas; the mesoderm has reached the anterior border of the area (fig. 15). Ten and three-quarter hours later, the formative area is slipper-shaped, the medullary plate is longer than the primitive streak; the first somite is in process of differentiating (fig. 19). Figure 16 shows this batch of eggs photographed fresh in utero (chart 2).

No. 486. January 29, 1921, eleven vesicles recovered, the normal ones measuring about 3 mm.; the primitive-streak stage, formative area pear-shaped, no medullary plate (fig. 17). Twenty and one-half hours later, embryos are in the 6-, 7-, or 8-somite stage. One of these is reproduced in figure 18 (chart 2).

No. 544. January 22, 1921, nineteen eggs removed, of which several are shown in figure 1. The vesicles are just a little earlier in development than those of no. 356. Seven days later, "large fetuses, 3 slightly hemorrhagic and one dead." These are no longer extant for examination. A photograph of the uterus with hernia of the mucosa pushed out by a fetus speaks for embryos of about the same stage as no. 549', shown in figure 2 (chart 1).

No. 560. This repeats the story of no. 486 almost exactly. The nine vesicles removed January 24, 1921, are slightly more advanced than those of no. 486, the mesoderm having spread beyond the limits of the formative area and the medullary groove having just begun (fig. 27). An eighteen-hour interval brought the embryos to the identical stage attained by 486' (fig. 18) (chart 2).

No. 561. January 24, 1921, eight eggs in the sixteen-celled stage (fig. 3, pl. 1). Seven and one-half days later, embryos with 7- to 9-mm. allantois, about two days of term. If in our chart the sixteen-celled stage is correctly placed at three and one-half days after ovulation, this period added to the interval of seven and one-half days gives us eleven days, or two days short of a thirteen-day gestation period for the embryo shown in figure 4 (chart 1).

No. 578. January 26, 1921, six vesicles, about 3.5 mm. in diameter removed. These have pear-shaped embryonic areas and long primitive streaks. Twenty-four and one-half hours later, the embryonic area was slipper-shaped, the embryo contained 6 somites (chart 2).

No. 580. January 27, 1921, eight vesicles, 4.0 to 4.5 mm. in diameter, primitive-streak stage. Both ovaries were removed at this time for experimental purposes. Four days nineteen hours later, the embryos had attained a stage approximately like those of no. 841. The specimen was not used in the charts.

No. 582. Nine 0.7-mm. blastocysts recovered, January 27, 1921. Considerable albumen is still left between the trophoblastic portion of the vesicle and the shell membrane (fig. 9). Both ovaries were removed at this time. Two days eighteen hours later, the vesicles (practically normal still) shown in figures 10 and 11, 8.4 to 9.0 mm. in diameter, embryos with about 6 somites were removed (fig. 12) (top of chart 2).

No. 585. January 28, 1921, young bilaminar blastocysts, 0.75 mm. in diameter, but slightly in advance of no. 582 and about the same as no. 293', were found. Left uterus and right ovary were removed. Five days less three hours later, 7.5-mm. embryos were recovered (fig. 6). Because of the similarity of the first

stage of this specimen and the second stage of no. 293, this interval has been added to that of 293 in chart 1.

No. 829. January 20, 1922, nine embryos with 2-mm. allantois. Both ovaries were removed at this time. Four and one-half days later, four embryos, slightly hemorrhagic, near term. This specimen is not utilized in the chart.

No. 838. April 1, 1922, oestrus was recognized in this animal for the third time. She was kept with a male. April 8th, thirty eggs were removed from one uterus, thirty-six from the other—a total of fifty-six—mostly excellent bilaminar blastocysts nearly 1.0 mm. in diameter. This is probably the record for normal ovulation in the history of mammalogy! In this case seven days' development postcoitum seems to be pretty nearly correct.

No. 841. January 21, 1922, removed left ovary and right uterus. Uterus contained fifteen ova (fig. 7) in late cleavage stages. Eight days fifteen hours later, four normal embryos within about two days of term (fig. 8) (chart 1).

No. 927. January 31, 1924, eggs in about the same stage as no. 544 (fig. 1): small vesicles with early polar differentiation. Both ovaries were removed with the left uterus. Eight days later, embryos with 3-mm. allantois. They are no longer extant for exact examination, though most like litter no. 549, from which the sketch shown in chart 1 was made (fig. 2). The embryos were still alive when removed, but may have been a little retarded because of the ovariectomy.

No. 931. January 29, 1924, removed right ovary and left uterus. From this eggs about like litter no. 294 were recovered (compare Hartman, '19, fig. 2, pl. 6, and fig. 1, pl. 11). Seven days later, embryos near term. The sketch in the chart was made from another litter near term, however, since embryos no. 931 were given away and no record of measurement could be secured from the recipient.

No. 969. February 7, 1925, ova in about the sixteen-celled stage. Seven days later, young embryos like those of no. 360 shown in figure 14.

APPENDIX B

PROTOCOLS OF ANIMALS WHOSE RECORDS FORM THE BASIS OF TEXT FIGURES G AND H

The protocols given in this appendix are presented in order that a definite idea may be given of the character of data upon which the first ninety days of postnatal growth is based. In no case, except no. 443, was a birth actually observed; but in a number of cases the birth was known within a few hours. A few cases are included in which an estimate of a week or ten days was made on the age of the pouch young when first discovered, and this involves some error, inasmuch as I had but a hazy notion as to the age of pouch young when this compilation was undertaken in 1927, two years after making the last collection of opossums. However, such pure estimates are only included in which the percentage of error finally is very slight, that is, the error of a day or two at the beginning of a long interval really constitutes an unimportant proportion of the total

length of time under consideration. Lengths are from snout-rump measurements. Where no sex is indicated, none had been recorded.

No. 116. February 1, 1916, pouch young just born. February 11th, eight young in pouch, two removed. March 2nd, removed a male pouch young (49 mm.; estimated weight, 4.5 grams) and a female (48 mm.; estimated weight, 4.5 grams); age, thirty-one days. March 14th, young measure 65 mm. (estimated weight, 7.0 grams). Young are hairy, testes well descended; age, forty-three days.

No. 517. Nine pouch young put down in the notes as ten days old, January 20, 1921. One measures 17.0 mm., another, 20.0 mm. The estimated weight of these is about 0.45 gram.

No. 528. Large female received January 22, 1921; by mammary palpation she was diagnosed as in late stage of pregnancy. July 9th, nine pouch young are found, of which six weigh 10.1 grams, or 1.7 gram each; length, 30 mm. The young are fifteen to eighteen days old. The age is plotted as of eighteen days, which seemed, all points considered, the probable age.

No. 552. Received January 25, 1921, in early stage of pregnancy. February 1st, one pouch young, about a day old. February 12th, young weighed 0.851 gram. Age of pouch young estimated at thirteen days.

No. 569. January 24, 1921, near oestrus; February 1st, pregnant. February 4th, one pouch young found, size not recorded, probably less than day old. February 19th, male pouch young weighed 0.65 gram; fifteen days old.

No. 591. February 5, 1921, pouch young 'about week old.' February 23rd, four young weighed: ♀, 2.35 grams; ♀, 2.20 grams; ♂, 2.29 grams; ♂, 2.29 grams; average, 2.3 grams; age, twenty-five days.

No. 593a. February 5, 1921, six pouch young, estimated at a little better than two weeks old. February 23rd, the young, all males, weighed 5.05, 4.97, 4.87, 4.87, 4.55, 4.43 grams. In alcohol they measure about 43 mm., perhaps equivalent to 45 mm. in life. Age, about thirty-four days.

No. 608. February 1, 1921, pregnant. Night of February 4th to 5th, parturition. February 10th, pouch young, a week old, observed, but not measured. March 11th, five young are left; these are killed and their heads removed for special brain fixation. Their preserved bodies are carefully compared with intact specimens and are found to be nearly the same size as batch no. 633, which weighed in the living state as follows: ♀, 3.16 grams; ♂♂, 3.52, 3.27, 3.21, 3.20, 2.92 grams; average, 3.22 grams. Five males measured about 37.5 mm.; age, thirty-five days.

No. 614. February 1, 1921, very small, red pouch young, not over a day old. February 10th, three 'good-sized' pouch young observed. February 20th, pouch young are dead; one weighed 1.5 gram. Age, twenty days.

No. 619. Large female, received February 2, 1921. Mammary palpation showed the animal to be in early stage of pregnancy, about ten days before parturition. February 20th, nine pouch young are seen; March 7th to 23rd, seven only; March 24th, the young, now forty days old, were killed. The weights were as follows: ♂♂, 7.15, 6.95, 6.51 grams; ♀♀, 7.60, 7.57, 7.01, 6.68 grams.

No. 624. February 4, 1921, received with pouch young at least a week old; February 16th, nine large young present. March 5th to 25th, six only left; March 26th, pouch young lost with the exception of one dead and one living. The latter weighed 8.5 grams; this was at least fifty-seven days old.

No. 636. February 5th, ten pouch young at least a week old. March 24th, the ten young weighed: ♂♂, 8.45 and 6.05 grams; ♀♀, 9.37, 9.25, 8.81, 8.44, 8.14, 7.89, 7.59, 7.19—an average of 8.8 grams for fifty-four-day-old pouch young.

No. 648. February 10, 1921, six pouch young about four days old. March 10th, the six young weighed 21.4 grams altogether—an average of 3.57 grams; length, 50 mm. Both mother and pouch young seemed rather lean. Age, thirty-two days.

No. 747. Received January 14, 1922. January 22nd, one pouch young two days old; January 28th, aged eight days; it weighed 0.64 gram.

No. 749. Pouch young born January 19, 1922. January 28th, at age of nine days, two were removed weighing 0.45 and 0.5 gram, respectively. February 2nd, at age of fourteen days, three pouch young weighed 0.81, 0.735, 0.61 gram; average, 0.722 gram. One female measured 23 mm.

No. 788. Received January 9, 1922, having been trapped, foot lacerated. Animal in early stage of pregnancy. January 16th, she was placed in a table drawer about 6 inches deep, where she gave birth during the night to a litter of ten young. Only one succeeded in attaching to a nipple; it weighed 0.16 gram. The other nine were dead and weighed together 1.2 gram, or an average of 0.133 gram each.

No. 824. January 20, 1922, received when in early stage of pregnancy. January 30th, mammary glands very thick and turgid, as when near term. March 24th, three pouch young, fifty-two days old, measure 68 mm. each and weigh 15.9, 14.9, 14.4 grams; average, 15.1 grams.

No. 861. January 22, 1922, pouch young two days old. February 20th, their weights and measurements were as follows: ♀, 4.23 grams; ♀, 4.02 grams; ♂, 3.98 grams (46 mm.); ♂, 3.95 grams; ♂, 3.52 grams; ♀, 3.33 grams; and two others, sex not recorded, each 3.9 grams in weight; average of all, 3.82 grams; age, thirty-one days.

No. 863. January 22, 1922, pouch young seventeen days old. February 1st, one pouch young, aged twenty-seven days, weighs 3 grams.

No. 876. Pouch young born January 30, 1922. March 24th, at the age of fifty-three days, one young was removed; its weight was 15.1 grams. At fifty-seven days (March 28th), five others were removed, weighing: ♂, 18.3; ♂, 18.0; ♂, 17.5; ♂, 16.6; ♀, 12.5; average, 16.6.

No. 883. Young born January 17, 1922. March 6th, at age of forty-eight days, two were removed; weights, 8.85 and 8.50 grams; length, 55 mm. At fifty-three days of age (March 18th), the following were removed: ♀, 15.6; ♀, 15.4; ♀, 14.9; ♂, 14.4; ♀, 13.4 grams. They measured 66 to 70 mm.

No. 884. January 24, 1922, eight pouch young were about five days old. Removed them February 4th. Their weights were as follows: 0.96, 0.94, 0.91, 0.91, 0.91, 0.9, 0.9—a very uniform series. Average weight, 0.92 gram; age, seventeen days.

APPENDIX C

Data concerning opossum pouch young 15 grams or more in weight, arranged in order of size.

No. 148. Length, 63 mm.; estimated weight, 15.0 grams. March 30, 1915.

No. 1002. Large litter, May 1, 1925. One young weighed 12.5 grams, a female, 15.0 grams.

No. 883. Fifty-three days old, March 18, 1922. Average weight, 14.7 grams.
1. ♀, 13.4 grams. Eyes closed; lips separated further on left side than right.
2. ♂, 14.4 grams. Eyes?; lips separated.
3. ♀, 14.9 grams. Both eyelids and lips separated.
4. ♀, 15.4 grams. Right eye open, left closed; mouth open.
5. ♀, 15.6 grams. Eyes closed; mouth open.
No. 824. Fifty-two days old, March 24, 1922. Average weight, 15.1 grams.
1. ♂, 14.1 grams. No description.
2. –, 14.9 grams, 68 mm., both eyes open in slits, lips separated.
3. ♂, 15.9 grams. One eye open, lips separated.
No. 876. Fifty-seven days old, March 28, 1922. Average weight, 16.6 grams.
1. ♀, 12.5 grams. Eyes open (slits); mouth open.
2. ♂, 16.6 grams. Eyes closed; mouth open.
3. ♂, 17.5 grams. Eyes closed; lips partly separated.
4. ♂, 18.0 grams.
5. ♂, 18.3 grams. Eyes closed; mouth open on one side only.
No. 139. 75-mm. pouch young (estimated weight, 16 grams), March 14, 1915.
No. 905. July 2, 1923, 85-mm. pouch young (estimated weight, 21 grams).
No. 907. July 17, 1923. Weights: 20.4, 21.1, 22.6, 23.5 grams. Eyes still closed, lips separated.
No. 150. April 17, 1915, 95-mm. (estimated weight, 25 grams) pouch young running freely about the mother. May 25th, as large as rats. Estimated age, one hundred days.
No. 998. March 25, 1925, pouch young the size of small mice, 95 mm. (estimated weight, 25 grams). Definitive hair color indicated.
No. 1007. May 14, 1925. A very uniform series. Eyes not yet opened, although the weights are much greater than others that had eyes open, as, e.g., nos. 824 and 876. Females: 27 grams, 102 mm.; 27 grams, 102 mm.; 28 grams, 104 mm.; 27.5 grams, 100 mm. Males: 26.5 grams, 100 mm.; 27.2 grams, 100 mm.; 27.5 grams, 100 mm.; 28.0 grams, 100 mm.; 28.0 grams, 103 mm. Average weight, 27.4 grams.
No. 1005. May 2, 1925. 30 grams. Eyes open.
No. 1003. May 1, 1925. 31 grams. Eyes open.
No. 940. April 27, 1924. A litter of eight, 45 to 51 grams (average, 48 grams) and 120 to 132 mm. (average, 126 mm.). This litter had been supplemented in nature by three larger young belonging to another female. The larger young weighed 101 grams and measured 170 mm.
Nos. 66 and 67. End of March, 1915. 138-mm. pouch young (estimated weight, about 55 grams).
No. 681. Caught May 25, 1921, in possession of two litters, her own of ten and a 'borrowed' one of about fourteen. Of the latter, two were said to have still had their eyes closed. This smaller litter escaped. The former litter ranged from 61.5 to 70 grams in weight; average, 66.6 grams. The mammary apparatus of the mother weighed 61 grams.
No. 1006. May 3, 1925. Weights and measurements as follows: 78.75 grams, 151 mm.; 80.5 grams, 152 mm.; 87.0 grams, 155 mm. The young were weaned, the mother's nipples greatly retrogressed. Two young wandered away from the mother and joined female no. 1005, which had 30-gram young.
No. 895. May 5, 1922: 1) ♂, 95 grams; 2) 95 grams, 158 mm.; 3) 157 mm.

No. 1009. June 14, 1925. The only litter of as many as fourteen pouch young that I have personally ever seen. With the mother, but no longer suckling. 1) 132 grams, 193 mm.; 2) ♀, 134.5 grams, 193 mm.; 3) ♂, 138.5 grams, 197 mm.; 4) ♂, 147.5 grams, 190 mm.

No. 1013. May 11, 1925. A litter of eight, average size, 145 grams, 185 mm.; except for one 'runt' of 92 grams, 140 mm. Range of the normal seven: 121 grams, 178 mm. to 165 grams, 203 mm.

No. 1001. This animal is remarkable for several reasons. She was a rather lean black female which, May 1, 1925, had six young the size of rats clinging to her and one small red pouch young, a day or two old, attached to a teat in the pouch; other nipples indicated the recent attachment of other newly born young that had been lost. The weight of the three large young: ♂, 140 grams; ♀, 155 grams; ♂, 155.2 grams. The color of the young was as follows: four were gray, the common phase of D. virginiana; one black, like the mother; one black, with a light sprinkling of white overhairs.

No. 939. February 15, 1924, caught by boys when the pouch young were 'the size of a pecan,' estimated by me to be at least twenty-one days old. When independent of the mother, the young were allowed to run free in the woods, but returned to the kitchen for food. April 28th, one of the young was brought to me, a male, 250 mm. in snout-rump length, aged not less than eighty-eight, nor more than ninety-five days.

No. 1008. Male, captured May 25, 1925; 290 grams, 244 mm.

APPENDIX D

Data concerning adolescent females, 920 grams or less in weight, in part entered in the chart, text figure A

PROTOCOL NO.	WEIGHT (GRAMS)	SNOUT-RUMP LENGTH	REMARKS
226	660	345	10.5-mm. embryos, February 11, 1915.
234	650	350	Pseudopregnant March 17, 1915.
275	503	310	Prepuberty, January 5, 1917.
276	601	325	Prepuberty, January 5, 1917.
278	651	320	Prepuberty, January 5, 1917.
281	590	290	Prepuberty, January 5, 1917.
321	900	375	Fetuses, January 26, 1917.
337	840	340	Fertilized eggs January 28, 1917.
339	900	350	Fertilized eggs January 28, 1917.
375	885	350	Fetuses, February 1, 1917.
474	867	—	First oestrus, February, 1921.
476	683	—	First oestrus, end of February, 1921.
497	920	297	Early prooestrum, January 10, 1921.
548	850	—	Immature, January 23, 1921.
671	—	'tiny'	7-gram pouch young, March 24, 1921.
684	373	245	Immature, May 31, 1921 (estimated age, 4½ months).
901	608	305	Immature, January 20, 1923.
1008	290	244	Immature, May 25, 1925.

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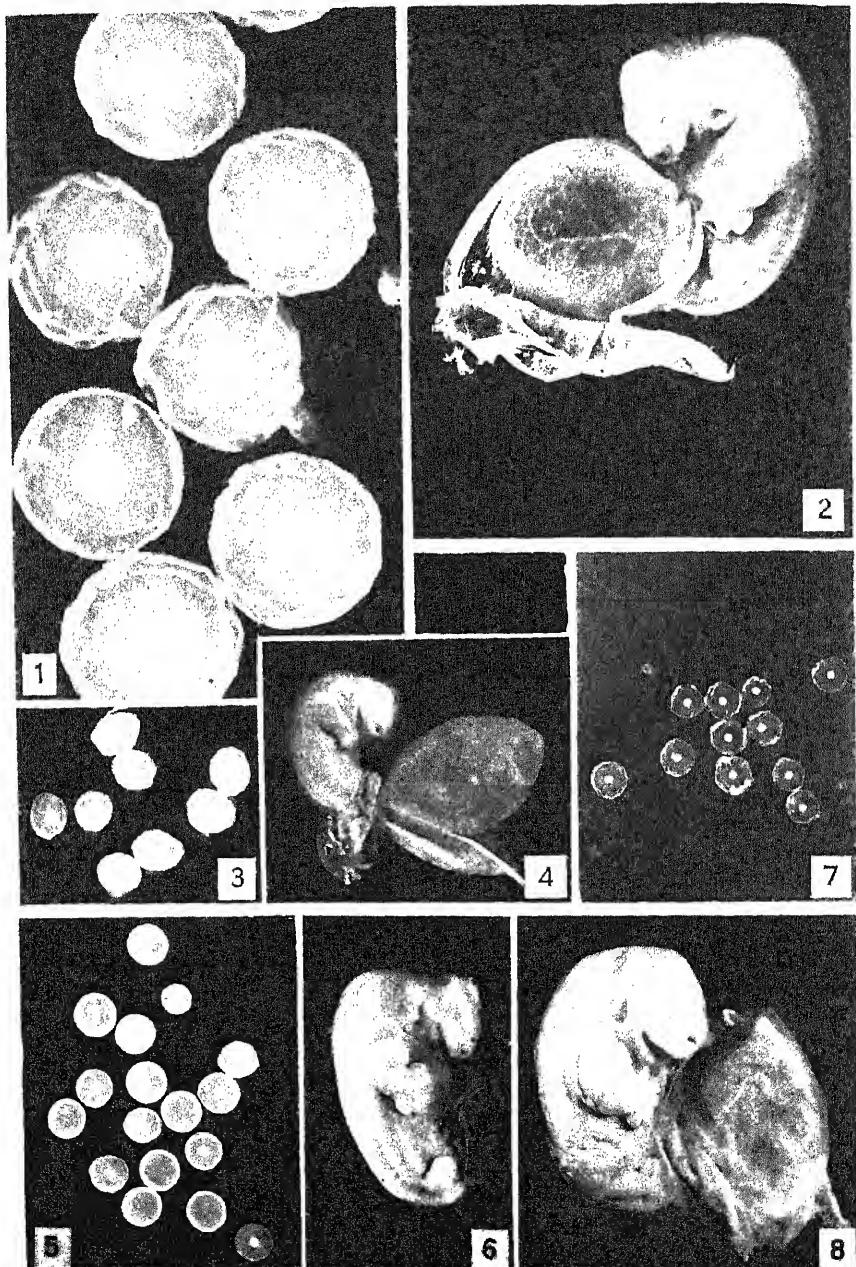
The eight figures in this plate illustrate four different developmental intervals as ascertained by successive removal of the two uteri of the same animal. In each of the four cases the embryos attained nearly the same stage of development at the end of the intervals. Figures 3 and 7 are cleavage stages of about sixteen cells ($\times$ 8); figure 1, small vesicles with evident polarity, early entoderm formation ($\times$ 40); figure 5, bilaminar blastocysts ($\times$ 8).

1 and 2　No. 544, seven days' development.
3 and 4　No. 561, seven and one-half days' development.
5 and 6　No. 585, five days' development.
7 and 8　No. 841, seven and five-eighths days' development.

9 to 12 Relate to no. 582. Figure 9, bilaminar blastocysts, × 8. Figure 10, the vesicles of the surviving uterus two and three-quarter days later, natural size; photographed alive in salt solution. One of the embryos with six somites is shown in figure 12, protographed by transmitted light in alcohol after cutting the lower hemisphere away (× 16). Figure 11, ova of no. 795 of the same stage as 582', showing the embryos on the surface of the vesicles as they appear alive in salt solution (× 3).

13 An embryo of no. 314', looking into the chorionic vesicle (× 4). The head of the embryo is flexed as in figure 14, protruding into the vesicle. Nine days postcoitum, five and one-half days after the early vesicular stage.

14 An embryo of no. 360, three days after the 1-mm. blastocyst stage. The proamnion has been removed to the vitelline vein, which can be seen just caudad of the fore-limb bud. The heart and pharyngeal clefts are well shown. No allantois is as yet visible.

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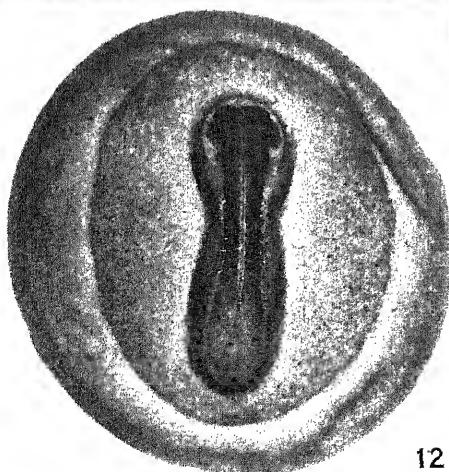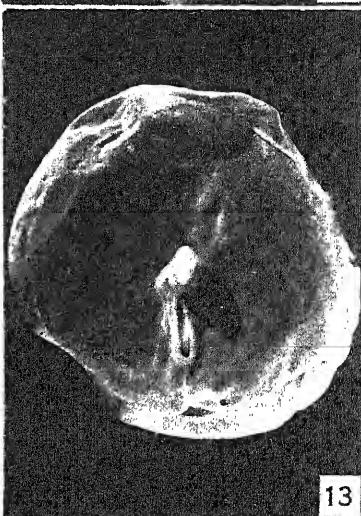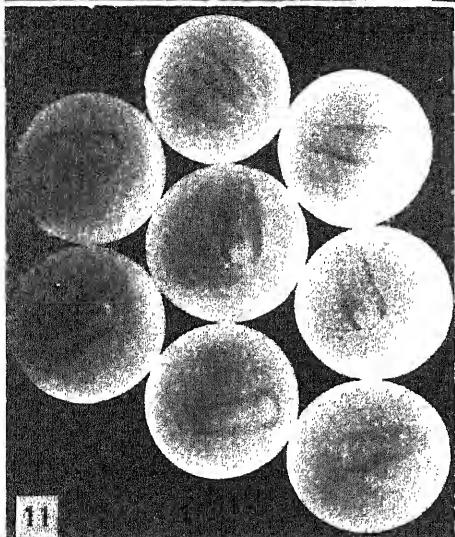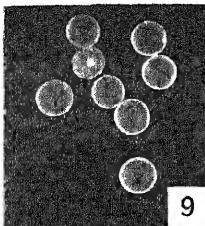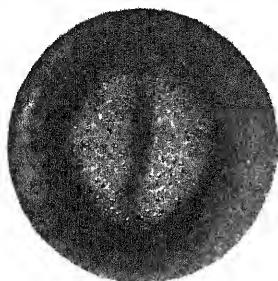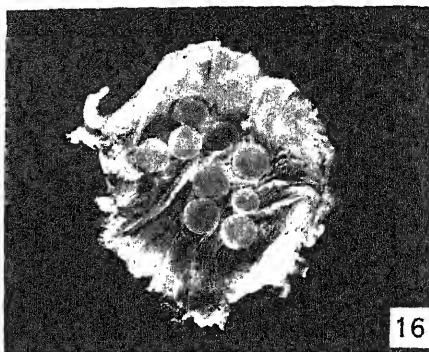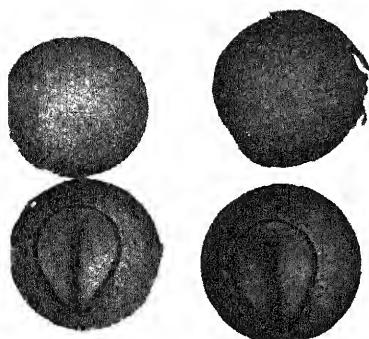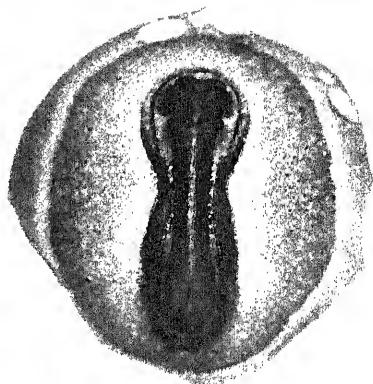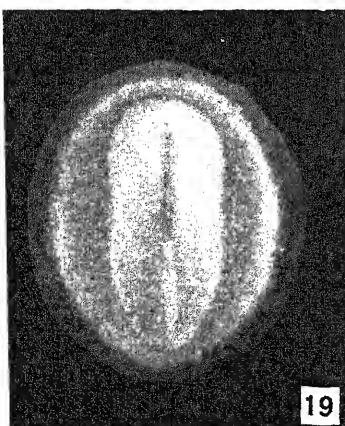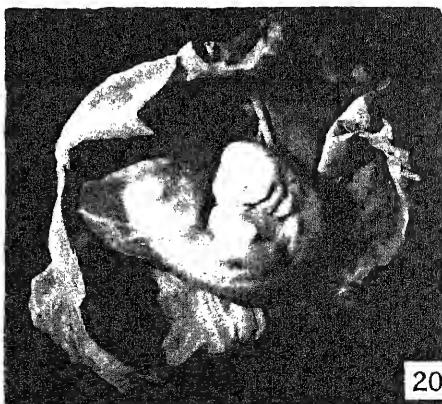
15, 16, and 19 relate to no. 380. Figure 15 is one of the eggs recovered at the first operation ($\times$ 16, photographed in alcohol by transmitted light). The egg represents the end of the 'proembryo,' the beginning of the actual embryo (beginning of the medullary plate). Yet in five and one-half days the embryo is destined to be born! In twenty and one-half hours the medullary groove has exceeded the primitive streak in length, as seen in figure 19. The embryo is slipper-shaped ('shoe-sole' embryo) and the first somites are about to appear. The photograph was made by reflected light. This egg is one of the litter shown in figure 16, photographed alive in the open uterus, natural size.

17　Two ova, formative and trophoblastic halves, of litter no. 486. $\times$ 16.

18　The stage reached twenty and one-half hours later ($\times$ 8). The limits of the mesoderm, the heart rudiment, somites, neural groove, and brain are well shown. The pair of lobes on the sides of the brain are strands of head mesoderm (compare Selenka, '87, Fig. 4, Tafel XX).

20　Embryo of no. 134', within its chorion. The anterior limb bud is well differentiated. A portion of the yolk sac covers the small allantois, ten days postcoitum.

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PLATE 4

Figures 21 to 29 represent altogether about one day's development, that is, from the seventeenth hour of the seventh day to the eighteenth or nineteenth hour of the eighth day (text fig. D). Text figure C gives the intervals involved in the eggs shown in figures 26 to 29. All photographs were made by transmitted light with the eggs in alcohol, the non-formative half of the eggs removed. $\times$ 16.

21 and 24 No. 346. In figure 21 egg 346(1) is still in the bilaminar condition; in figure 24 the egg, 346'(2), has the mesoderm occupying an elliptical area about the primitive streak; the embryonic area is beginning to elongate.

22 and 23 Eggs 353'(3) and 353'(5). The former has twenty-seven mesodermal cells; the latter about 125, seen as a cloud in figure 23.

25 Egg 338(9). The spread of the mesoderm is well shown. No time relations are known for this egg.

26 Egg 337'(1), four and one-half days after the sixteen-celled stage. A small mesoderm-free area still persists at the anterior margin of the embryonic area.

27 Egg 560(1). Mesoderm has spread beyond the margin of the embryonic area; medullary groove is hardly begun.

28 Egg 344'(2). A short medullary groove is seen. This stage was attained (text fig. C) nearly three days after a late cleavage stage.

29 Egg 298'(3). Eighty-six hours after the early vesicular stage, i.e., after cleavage had just ended. Note the limits of the mesoderm, the embryonic area, the primitive streak, the medullary groove (light area), and the notochord (shadow under the medullary groove).

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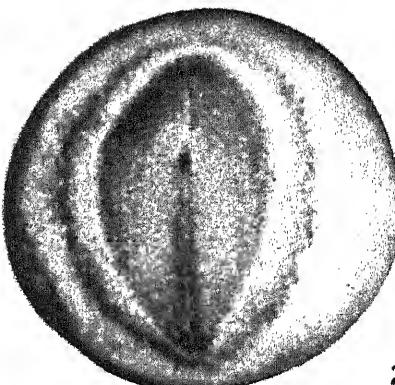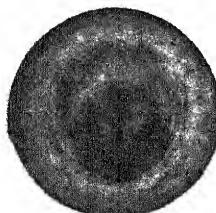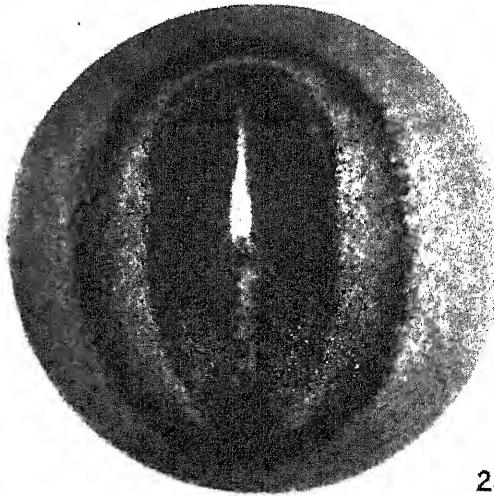
SPERMATOGENESIS OF BRUCHUS QUADRIMACU-
LATUS (COLEOPTERA: BRUCHIDAE)

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SIXTY-ONE FIGURES

AUTHOR'S ABSTRACT

Notwithstanding the fact that several species of Bruchidae have been used by geneticists for several years, no cytological studies have as yet been made on any member of this family of the Coleoptera. The present paper gives a general account of the spermatogenesis of Bruchus quadrimaculatus Fabr.

The spermatogonia undergo two mitotic divisions. After the second division, the nuclei remain small and very dense for some time before the beginning of the growth phase. During this interval the nuclei do not assume again the characteristics of the interkinesis stages. In the primary spermatocytes typical tetrads are formed. The chromosomes are asymmetrically V-shaped. The end of one arm of the 'V' fuses with the end of the corresponding arm of its synaptic mate. Disjunction takes place in the primary spermatocyte division. After the division of the secondary spermatocytes, the chromosomes become vesicular and form a reticular nucleus in the spermatid. after which the chromatin becomes deposited as a chromatin rim around the nuclear periphery. The diploid number of chromosomes is nineteen in spermatogonia and in male somatic cells, and twenty in female somatic cells. An unpaired X chromosome is present in the spermatogonia, which fails to divide in the primary spermatocyte division, but passes as a whole to one pole in advance of the autosomes. The X chromosome divides normally in the secondary spermatocyte division with the autosomes.

In the method of sex determination, Bruchus does not follow the method of the majority of beetles, since most of those studied adhere to the X-Y type.

Although the beetles have received considerable attention from cytological investigators, there still remain families upon which no cytological studies have been made. One of the latter is the family Bruchidae, which in recent years has been employed by geneticists for work in this field. Bruchus quadrimaculatus has been used by J. K. Breitenbecher for about nine years. Several Bruchidae, but chiefly B. chienensis, were employed by S. H. Skaife ('25). The writer (Brauer, '25) reported on the embryological development of B. quadrimaculatus, but made no attempt at a study of the germ cells at that time. Brooks ('22), in a 'Preliminary note' on the chromosome number of the first spermatocyte of B. quadrimaculatus, made a tentative estimate of the number of chromosomes from polar plates of the primary spermatocyte

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spindles. This report, however, was in itself so meager in details and based upon such scanty evidence, that the number was still doubtful. In the present paper I suggest that his figures illustrating the reductional division are questionable. The problem of chromosome behavior in spermatogonia and spermatocytes, chromosome number in these cells as well as the method of sex determination, were left untouched.

In this paper it is the writer's purpose to give a general account of the spermatogenesis of B. quadrimaculatus, Fabr., from the early spermatogonia to mature spermatozoa, as well as to demonstrate the chromosome number in spermatogonia, primary and secondary spermatocytes and in somatic cells of both sexes. The behavior of the sex chromosomes will, of course, receive attention.

METHODS EMPLOYED

Since the beetles are readily incubated in the laboratory, lack of sufficient material of the proper stages is not a factor in this problem. The chief difficulty is that the beetles are small in the adult stage and smaller still in many of the critical larval stages, so that methods of obtaining gonad sections had to be devised. The insects were incubated at a constant temperature of 30°C. and in a very nearly saturated humidity. The chronological development under these conditions is briefly as follows. Embryonic development of larva in the egg requires five days. Larval life in the cow pea requires nine days. Pupal life to the time of emergence occupies five days. The entire cycle consequently from oviposition to emergence is completed in nineteen days. Since the insect emerges as adult, the females begin to deposit the eggs within a few hours after emergence. In speaking of pupae removed on the fourteenth day, we have in mind the entire nineteen-day period of development, and the degree of development is practically constant for these conditions.

The maturation cycle of the germ cells begins at approximately the eighth day and is practically completed by the sixteenth day, or about two days before emergence of the

adult. The entire abdomens of the removed larvae and pupae were prepared by standard cytological methods and the abdomens served as a guide for exact orientation of the gonads for sectioning. Some of the best spermatogonial and spermatocyte phases were seen in smears, and it was from these that many of the drawings were made. Embryos of twenty to thirty-six hours' incubation were employed for chromosome counts in somatic cells.

Each testis is a bipartite body lying in the center of the posterior part of the abdomen in the sixth, seventh, and eighth segments, lateral to the intestine. These bodies are well differentiated by the eighth day of incubation. There is apparently no difference in the degree of development of the germ cells of the two testicular parts, but some of the cysts in each of these parts may be considerably more advanced than others. The cysts in the periphery of the testes are usually in advance of those nearer the center of these bodies, and this relationship is maintained until they become mature spermatozoa.

THE SPERMATOGONIA

The nuclei of the spermatogonia of the eighth day have attained their greatest size. Their average diameter is at this time 5.7 μ. The chromatin lies in irregular masses and appears to be peripherally disposed, while the linin threads nearer the center contain relatively little (fig. 1). Some nuclei show a large dense mass of this material, which may be the intensely stained body which in spermatocytes is recognizable as the sex chromosome. Since the chromatin at this time lies on the nuclear periphery and because the center is correspondingly clear, the nuclei are well defined. Mitotic divisions are not uncommon among spermatogonia of the eight-day larvae in regions showing more advanced stages of development (figs. 5, 6). In the cells of cysts adjacent to those in which mitosis is in progress, the spermatogonial nuclei appear more like those of figures 2, 3, 4. The chromatin becomes more evenly distributed over the linin network to

form a continuous spireme. A condensation follows this and then a segmentation into chromosomes (figs. 4, 5). In the former figure the chromosomes are still long and lie in various focal planes. This is followed by a rapid condensation in which the various shapes assumed by the chromosomes can be seen. Nuclei in which the full spermatogonial number can be counted at this time are not common, because of the difficulty and uncertainty of counting them when they lie at different levels. From metaphase plates, however, of the first spermatogonial division, the number was determined to be nineteen (fig. 8). Morphologically, a number of the chromosomes are seen as irregular 'V's,' sometimes sharply bent and sometimes rounded into 'U's.' In general, they correspond in shape with those of other coleopterous families. The chromosomes, however, show considerable variation in shape in the cells of different individuals, and even in different cells of the same individual. The chromosomes are seen entering a spindle in figure 5.

The metaphase and anaphase of the first spermatogonial division resemble corresponding stages of somatic mitoses. The chromosomes are closely massed and proceed to the poles in the same plane. Each polar mass is surrounded by a considerable amount of uniformly staining cytoplasm which stains less intensely than it did preceding the division.

From the tenth to the twelfth day, the spermatogonia undergo their last divisions, and by the end of this time some have begun their growth as primary spermatocytes. It is not unusual to find in larval testes of eleven to twelve days, in practically all of the cells, the dense chromosomal clumps that mark the completion of this division.

The last spermatogonial division is apparently not preceded by the elaborate prophases that precede the first division, but seems to succeed this division immediately. The telophase nuclei of the first division do not assume again the characteristics of the resting stages described for the former division, but the chromosomes remain intact, contracting somewhat to enter the spindle of the last spermatogonial

mitosis (figs. 7, 9). Here the chromosomes are short. Some of the smaller ones are almost spherical. The cytoplasm is very clear, so that the cells after division appear almost devoid of it (figs. 15, 16, 17). In the telophase the chromosomes again lengthen, but, until the cells begin their growth, these dense nuclear clumps remain surrounded by the clear cytoplasm and the distinct cell membrane.

In the spermatogonial divisions the chromosomes, as a rule, lie in the same plane during the various mitotic phases. In metaphase they form an almost perfect plate (fig. 10), and in the successive stages advance toward the poles, and come into closer and closer contact as they near the poles. Figure 11 is an unusual division, for the reason that the pair of chromosomes lying lateral has apparently divided precociously and off the equatorial plane.

Since all of the chromosomes usually lie in the same plane in the spindles, unusually good polar views of metaphase and anaphase plates are often encountered in eleven- and twelve-day larval sections. The shape of these spindles in transverse sections is imperfectly elliptical or oval (fig. 13), but it may be polygonal (fig. 12). In these figures twelve or thirteen chromosomes lie in the outer ring and six or seven, as the case may be, occupy the center of the figure. An almost invariable count in plates shows nineteen chromosomes on the spindle, and when these are compared to the perfect plates seen in profile there seems little probability that any have been excluded from the picture.

PRIMARY SPERMATOCYTES

Following the secondary division of the spermatogonia the nuclei are small and surrounded by little cytoplasm (fig. 14). Their average diameter is now but 4.7 μ and they are dense with the heavily staining chromosomes of the preceding telophase (fig. 15). This stage is very characteristic in Bruchus. After the last spermatogonial division, there appears to be a lapse of time before the spermatocyte nuclei begin their period of growth. Frequently, in larval sections

of eleven and twelve days, practically all of the cells in the testes are in this condition, with relatively few that have begun their growth. The chromosomes of these dense nuclei elongate, but retain their staining reactions (figs. 16, 17). They are irregularly arranged and retain their characteristic shapes to some degree. Frequently, as many as fifteen may be counted. Goldsmith ('19) describes a condensation at the spermatogonial telophase, which, however, does not appear to continue in the spermatocyte nuclei to the extent that it does in Bruchus. Nowlin ('06), on the other hand, states that the telophase nuclei pass immediately into the synaptic knot, in Coptocycla, and she shows no intervening stages. Hayden ('25) has carefully shown the growth phases of the spermatocytes for the beetle Phanaeus, but here the telophase nuclei of the spermatogonia apparently pass immediately into the leptotene thread. According to Nonidez ('20), in Blaps, the nuclei after the last spermatogonial division again become typical resting nuclei with interkinesis characteristics. Stevens ('06, '09) has not described presynizesis phases for the many beetles with which she worked, so that it is not possible to tell whether this stage is characteristic for other Coleoptera.

From the twelfth to the thirteenth day, the nuclei begin their growth. The chromosomes elongate to form a leptotene thread (figs. 18, 19, 20). Others are found in synizesis (figs. 21, 22). At the time synizesis occurs the nuclei are large and the synaptic knot occupies only a part of the nucleus. In some nuclei this seems to be a formless tangle (figs. 21, 22), while in others there is a more definite arrangement and orientation of the loops. With the loosening and lengthening of the loops, this is even more apparent (figs. 23, 24). In postsynizesis nuclei the diplotene threads are loosely arranged and feathery (figs. 25, 26). In many of them a longitudinal split is apparent (figs. 27, 28). The threads contain a number of densely staining bodies, and a plasmasome may be seen. In sections the nuclei at this time have an average diameter of 7.8 μ, which is the largest size attained by the

male reproductive cells. In smears they are somewhat larger (fig. 29). An increasing tendency to stain densely can be noted as the threads shorten (figs. 30 to 32).

THE SEX CHROMOSOME

The sex chromosome could definitely be distinguished for the first time in the metaphase plate of the first spermatogonial division (fig. 8). Here it appears as an elliptical body, whereas the autosomes have the form of 'V's.' It remains more nearly uniform throughout the various cell generations and throughout the cycle of the same cell than do the autosomes. During the mitotic division of the spermatogonia the sex chromosome divides with the autosomes in the metaphase plate, and after division the two parts of the divided chromosome remain in line with the others.

During synizesis this body cannot usually be distinguished in the spermatocytes, but during postsynizesis the chromosome is again visible as a sharply defined elliptical body (figs. 25, 29). Here the chromosome stains densely and appears to lie in an individual vesicle at the periphery of the nucleus. When viewed from the side, there appears to be a bulge in the nuclear membrane because of this vesicle (fig. 25). The plasmasome can usually be readily distinguished from the sex chromosome, as it destains much more readily than the latter and lies deeper in the nucleus.

THE TETRADS AND THE PRIMARY SPERMATOCYTE DIVISION

Excellent tetrad groups were found in smears of primary spermatocyte nuclei just preceding the first maturation division (figs. 33, 34). The tetrad type that is common in beetles is found in Bruchus. The chromosomes are in the form of asymmetrical 'V's' and the longer arm of the 'V' fuses with the corresponding arm of its synaptic mate, giving the bivalent the form of a double hook, or occasionally, when seen in profile, the form of an 'E.' The longitudinal groove in the fused chromosomes is at this time very conspicuous, so that actual tetrads are produced.

The tetrads become arranged on the spindle in the first maturation figure with their longitudinal axes parallel to the long axis of the spindle. The spindle fibers are attached to the apex of the 'V' and the fusion point of the 'V' arm lies on the equator of the spindle (figs. 35, 36). As disjunction takes place the fused arms are often drawn out enormously before separation takes place (fig. 37). In one pair of 'J's' the long arms are fused with the hooks turned in opposite directions (figs. 33, 37). This, however, seems to be an unusual synapsis. The X chromosome, having no synaptic mate in this division, is drawn to one pole peripheral to, and in advance of, the disjoined autosomes. In cross-section the spindles are oval or circular in shape. Seven or eight chromosomes lie in the outer ring and one or two members are usually found in the center. The X chromosome lies in the outer ring, occasionally even outside the outer circle (figs. 38 to 41). If the spindle is sectioned as the chromosomes approach the poles on the side of the spindle containing the X member, all of the chromosomes may be in the same focal plane, while in sections near the equator the X chromosome will be dimly in focus or entirely out of the picture. Frequently, in polar views when all of the chromosomes are in the same focal plane, it becomes difficult to distinguish between the X chromosome and autosomes.

THE SECONDARY SPERMATOCYTE DIVISION

The secondary division of the spermatocytes follows the first immediately. The haploid chromosomes become arranged on the spindle with the longitudinal groove on the equator of the spindle (fig. 42). In the figures containing the X chromosome this member again usually lies peripheral to the autosomes, as it went into the telophase in the first spermatocyte division (fig. 49). The X chromosome now divides into two equal parts which pass poleward with the autosomes. Here again the chromosomes of each group all lie in the same plane. The spindles are shorter than those of the primary spermatocyte, but are relatively broad in the

metaphase (figs. 43, 44). In smears, half or more of the chromosomes may be visible in profile views. A considerable amount of cytoplasm still surrounds each pole and this often appears fibrillar (figs. 45, 46). Chromosome counts made from a large number of transversely cut spindles showed that approximately half of these contained nine, and the remainder contained ten chromosomes (figs. 45 to 48).

In the telophase of this division the chromosomes appear to swell and stand out as distinct and separate elements even in profile view (fig. 50). A tendency to become vesicular, with a loss of staining ability, begins to manifest itself in the central portion of each chromosome. Slightly later, this is seen distinctly in polar views (figs. 51, 52). Here the chromatin becomes aggregated on the periphery of each of these chromosomal bodies, and they become closely appressed and give to the entire nucleus a reticular appearance, with a mesh representing each chromosome. Nine of these vesicular bodies were counted in many of the nuclei. There now becomes apparent a tendency for the chromatin to become arranged on the nuclear periphery, so that the effect is to produce a nucleus that is clear in the center, with a rim of chromatic matter just inside the nuclear boundary (fig. 53), but with the reticular threads in the center of the nucleus still containing traces of chromatin. The threads at one side of the nucleus now break and seem to be pressed or drawn to the periphery, often leaving the center of the nucleus very clear (fig. 54).

I fail to find in the literature of coleopteran spermatogenesis the description of a spermatid nucleus of this kind, but probably a near approach of it is found in the spermatid nucleus of Squilla oratoria: Crustacean (Komai, '20). Here the chromosomes of the telophase of the secondary spermatocyte division form a compact chromatin mass, according to the author, until it gives the nucleus a reticular appearance. After the nucleus has maintained this structure for some time, some of the reticular meshes coalesce to produce a large mesh which presses the remaining part of the network

against one side of the nucleus into a crescent-shaped mass. The author does not state whether the number of meshes which appear in the chromatic mass have their origin in each of the chromosomes or whether their number corresponds to the chromosome number. The nuclei to some extent seem to resemble those of Bruchus, excepting that the meshes are not nearly so regular. In Squilla the chromatin is carried to the nuclear periphery only after the coalescence of some of the meshes, to produce a crescent-shaped mass, while in Bruchus the chromatin is deposited on the nuclear periphery while the network in the center of the nucleus is still very much intact.

The nuclei now begin to stain more densely, but continue to destain from the center outward. The cytoplasm becomes distinctly fibrillar. A clear area first appears around the nucleus and advances as the cytoplasm elongates and becomes narrower. The axial filament courses through the cytoplasm from a small prominence at the base of the nucleus and projects from the lower end of the fibrillar cytoplasm (figs. 55 to 57).

It has not been possible to distinguish the spermatids which carry the sex chromosome from those which do not in the stages described above. Attempts to identify it in the vesicular stage were not successful.

The spermatozoa are long slender threads in which it is scarcely possible to distinguish different areas. In figure 58 there appears a small clear area at the tip of the head and slight irregularities in the middle-piece region. The mature spermatozoa have a length of 130 μ and in normal salt solutions show a sluggish motility.

SOMATIC MITOSIS

Somatic cells showing chromosome morphology and number were selected from ectoderm, entoderm, mesoderm, or serosa of embryos of twenty-eight to thirty-six hours' incubation where mitotic divisions are numerous and the cells are clear and distinct.

In these cells the chromosomes again appear as asymmetrical 'V's' as they did in the spermatogonia, but are extremely variable in shape in different individuals and in different cells of the same individual. In the metaphase plate the vertex of the 'V' is directed toward the center of the spindle and the concavity is directed outward. The chromosomes of the outer ring form a circle. The members of the pairs usually lie near each other, but as in other insects they frequently do not.

Chromosome counts from cells of some embryos showed twenty chromosomes with a pair of X chromosomes. The embryos with this pair of bodies may, of course, be designated as potential females (figs. A, B). Cells of other embryos
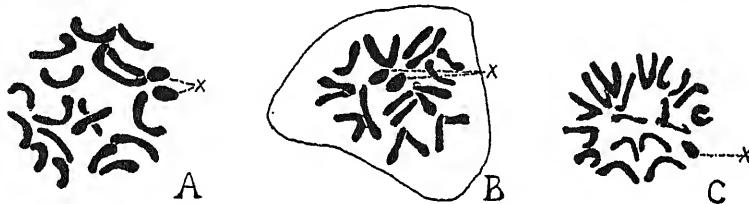
Figures A, B, and C

contained only one X, and were designated as potential males (fig. C).

Attempts to obtain desirable polar plates of pole cells proved unsuccessful, although much time was spent in trying to do this. Several views were obtained in which as many as fifteen could be seen.

DISCUSSION

The behaviors of the sex chromosomes of the beetles have been grouped into three different types.

In type I are placed those which possess an unequal pair of sex chromosomes, commonly known as the X-Y type. According to Harvey ('16, '20), thirty-five of the seventy-two species listed were of this type, but the method of sex determination was not given in many of the beetles studied.

Stevens found this type in thirty of the forty-five species which she studied. The thirty species represent fourteen families.

Type II has a bivalent X chromosome which passes undivided to one pole in the first division, but divides normally in the second division. In the forms in which this type is found both the male and the female somatic cells have an even number of chromosomes, but the female has two more than the male. This type was described for Leptinotarsa by Wieman ('10), and for several Ceccindelidae by Goldsmith ('19).

Type III is characterized by the single odd chromosome in the male cells and is commonly known as the X-O type. This type seems to be much less common in beetles than is the X-Y type. Prior to this paper, this type was found to occur in thirteen species representing seven families of Coleoptera. The chromosome behavior of ten of these species was reported by Stevens. In all of these species the unpaired X passes entirely to one pole in the primary spermatocyte division and divides with the autosomes in the secondary spermatocyte division. The individual differences in its behavior need not be recounted here.

This paper shows that Bruchus belongs to type III. Whether B. quadrimaculatus, like Chrysomela similis and Diabrotica vittata (Harvey, '16), are exceptions to the type occurring most commonly in the family or whether the unpaired X is the rule in the family as it is in the Elateridae and Lampyridae, is not known.

Brooks ('22) was able to demonstrate polar views of the first spermatocyte division for Bruchus, in which he showed plates containing, respectively, nine and ten chromosomes. From these views he surmised that ten was the haploid number of chromosomes. His figures illustrating the polar views of this division are similar to my figures 38 to 41. In regard to his illustrations of the primary spermatocyte division, in which he illustrates the disjunction of the chromosome pairs, Brooks is clearly in error, for in no wise do his figures show

the form of the tetrads nor the manner of their reduction. The figure is problematical. If drawn on the same scale as his other figures, it might be taken for the secondary spermatocyte division, were it not for the undivided X chromosome which is featured. His figures of the anaphase of the same division (figs. 5, 6) do not agree with the former (fig. 3). In his figure 6 entirely too many elements are seen in this kind of view for an anaphase of the primary spermatocyte division. Figure 6 might well be mistaken for an anaphase of the spermatogonial division, or even for a somatic mitosis. In the primary spermatocytes the chromosomes are unmistakably of the form of asymmetrical 'V's,' each with a longitudinal groove, and synapsis takes place as illustrated in my figure 33. Brooks' figures make it decidedly questionable as to whether he actually saw the spermatocyte divisions or mistook for these the mitotic divisions of other cells.

SUMMARY AND CONCLUSION

1. The behavior of the autosomes in the germ cells of Bruchus quadrimaculatus during the spermatogonial, and first and second spermatocyte divisions agree in general with those of the other beetles which have been studied.

2. Following the last spermatogonial divisions, there is a lapse of time before the spermatocytes begin their growth phases, but during this time the spermatogonial nuclei of the last division do not assume again the resting, or interkinesis stages of early spermatogonia, but the chromosomes retain the characteristics of the spermatogonial telophase. The leptotene thread is formed with the beginning of the growth period.

3. Synizesis takes place when the primary spermatocytes have completed their growth. The manner of orientation of the loops is scarcely recognizable till the loops begin to lengthen postsynizesis.

4. An unpaired X chromosome is present in spermatogonia which in the primary spermatocyte division passes to one pole undivided, in advance of the autosomes which are undergoing their reductional division.

5. The secondary spermatocyte division is an equational division for the autosomes and for the X chromosome in the cells which carry it.

6. The telophase of the secondary spermatocyte division is marked by the appearance of a syncytium, the meshes of which develop from the chromosomes.

7. The number of chromosomes for the various cells of Bruchus is as follows: spermatogonia, nineteen; primary spermatocytes, nine tetrads and one unpaired chromosome; secondary spermatocytes, nine and ten; somatic cells of embryo, nineteen and twenty.

8. The manner of sex determination found in Bruchus is the type of the minority of the beetles studied; the majority belong to the X-Y type.

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EXPLANATION OF FIGURES

All figures drawn with camera lucida. Initial magnification, 1500 diameters. Enlarged to 3000 diameters and reduced one-third in printing.

PLATE 1

1 Eighth-day spermatogonia from a single cyst.

2 to 4 Spireme stages preceding first spermatogonial division.

5 Prophase of first spermatogonial division.

6 Telophase of first spermatogonial division.

7 and 9 Spermatogonial nucleus after first mitotic division.

8 Metaphase plate of first spermatogonial division.

ALFRED BRAUER
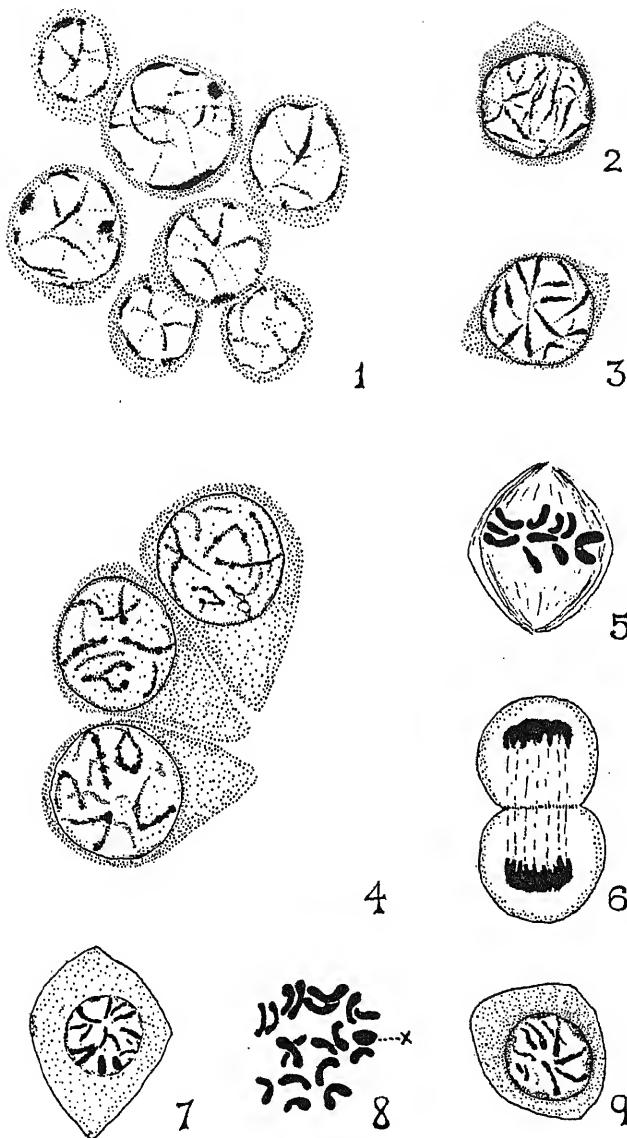
10 and 11   Metaphase and anaphase respectively of second spermatogonial division.

12 and 13   Metaphase plate of same.

14   Late anaphase of second spermatogonial division.

15 to 17   Spermatogonial nuclei after the second mitotic division and preceding growth phases.

18 to 20   Growth phases of primary spermatocytes. Leptotene nuclei.

21 and 22   Synizesis.

23 and 24   Post synizesis. Loosening of chromatin network.

25 and 26   Diakinesis.

PLATE 3

ERRATUM

A COMPARATIVE STUDY OF THE SPERMATO-GENESIS OF FIVE SPECIES OF EARWIGS[1]

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EIGHT FIGURES

AUTHOR'S ABSTRACT

The following species of earwigs are used in this study: Labidura bidens, Labia minor, Anisolabis annulipes, Anisolabis maritima, and Forficula auricularia.

1. In all species the chromosomes are divisible into, *a*) autosomes and, *b*) XY-complex.

2. The chromosome distribution is regular in Labidura bidens and Labia minor. The male diploid number is 12 and 14, respectively. Each has an XY-complex in which the X is a single chromosome.

3. In both Anisolabis annulipes and Anisolabis maritima the male diploid number is 25, or 22 autosomes and an XXY-complex. The two X components remain fused during the first spermatocyte division.

4. The diploid number in the male of Forficula auricularia is 25 and 24. The chromosome number is constant in the individual. The irregularity is interpreted as due to the fusion of the two X components in the individuals with 24 counts and to these X components remaining separate in the earwigs with the 25 counts.

5. An explanation is given for some of the variable results obtained in former studies of the chromosomes of Forficula auricularia.

6. The discussion considers the possible origin of the variations in chromosome numbers in the earwigs.

INTRODUCTION

The following comparative study of the chromosome behavior in the earwigs was begun in 1920, and a preliminary paper on Anisolabis annulipes was reported in 1922. Much difficulty has been encountered in obtaining specimens of certain species in sufficient quantities to complete their study.

Beginning with Carnoy, in 1885, several cytological studies have been made on the European earwig, Forficula auricularia. Following the work of Carnoy, the other European cytologists reporting studies on this species were St. George ('87), Sinety ('01), and, finally, a more detailed study in two papers by Zweiger, in 1906. In this country Stevens ('10) and Payne ('14) have contributed to the cytological literature of Forficula auricularia. Although the above

[1] Contribution from the Zoölogical Laboratory of Indiana University, no. 214. Submitted in partial fulfillment of the requirements for the degree, Doctor of Philosophy.

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papers have dealt primarily with the chromosomes, three other workers might be mentioned here: Pantel ('12), Brauns ('12), and Meeks ('13). These workers, though directing their studies toward other phases of cytology, have incidentally covered the subject of chromosome distribution, but added little toward reconciling the diverse observations of previous workers. In a short paper, Payne has shown that the disagreement of the former observers was due, at least in part, to the great individual variation found in the behavior of the chromosomes of this species. In spite of this great mass of conflicting observations and explanations, it is hoped that the present paper, through a comparative study with other species, will make the conditions in Forficula auricularia more understandable.

The earwigs native to this country seem to have been neglected. Randolph's paper in 1908 on Anisolabis maritima was the first report. This species has been more recently reworked by Kornhauser and several inaccuracies corrected.

Although the above summary of the literature has been stated only topically, it is sufficient to show that there has been disagreement in the results of these workers. Space has not been taken in the present paper to point out the inaccuracies in observation and explanation of former workers.

The present investigation was made under the direction of Dr. F. Payne, to whom I am indebted for helpful criticism and advice and for his many splendid preparations of Forficula auricularia which have been used in this study.

MATERIAL AND METHODS

Earwigs for this study were collected in several parts of the United States (Florida, Alabama, Mississippi, New York, Indiana, California, and Louisiana), Zürich, Switzerland, and the Bahama Islands. They were in various stages of development, from young nymphs to old adults. The material was identified by A. N. Caudell, of the Bureau of Entomology, and

James A. G. Rehn, of the Philadelphia Academy of Natural Sciences.

Various fixing and staining methods were tried, but the bulk of the material was fixed in Flemming's fluid (strong) and stained with Heidenhain's iron-hematoxylin. Some of these were counterstained with light-green or eosin. A small amount of the material was fixed with Benda's modification of Flemming's fluid and stained with alizarin and crystal violet. Smears of testes were made of Labidura bidens, Anisolabis annulipes, and Anisolabis maritima, but this material offered little advantage over sections for the study of chromosomes.

In each species, except Labia minor, a great mass of material was fixed and sectioned so that the intensive part of the study was made from only the best of numerous excellent preparations. The large amount of material examined, especially in Forficula auricularia, has offered excellent opportunity to avoid inaccuracies and to check individual variations. The European material was procured and prepared by Doctor Payne.

The drawings were made with the aid of the camera lucida, using a 1.5-mm. Zeiss apochromatic objective and 10× compensating oculars on a 7J Spencer microscope. After the usual correction, these drawings were enlarged to twice the original size and again corrected, so that the figures are believed to be essentially accurate. The magnification of the finished figures was about 3800 diameters. In reproduction the drawings were reduced to about 2500 diameters.

DESCRIPTION

Labidura bidens Oliv.

Specimens of this species were collected in the locality of St. Petersburg and Dundee, Florida, New Orleans, Louisiana, and Bahama Islands. Most of the material came from the first-mentioned locality.

By counting the clear metaphase plates of the spermato-
gonia of many individuals, the diploid number of the male
was found to be 12 (fig. 1, A, B, and C). Several oogonial
counts showed the female number to be the same as the
male (fig. 1, D). Due to differences in size and shape, the
homologous pairs could be easily distinguished in the best

Fig. 1 A, B, C, spermatogonial metaphase with 12 chromosomes; D, oogonial
metaphase with 12 chromosomes; E, homologous pairs of oogonial chromosomes;
F, spermatogonial chromosomes grouped in homologous pairs; G, H, first
spermatocyte polar view, showing 6 chromosomes; I, group of unequal pairs
of first spermatocyte; J, all the chromosomes of a single cell in side view; K,
L, second spermatocyte metaphase with 6 chromosomes.

preparations (fig. 1, E, F). In comparing the individual
spermatogonial and oogonial chromosomes it was seen that
the diploid female group lacked one small chromosome found
in the male group. In the spermatogonial chromosomes two
individuals could not be paired. One of these was similar
to the chromosomes of one of the oogonial pairs, but the other
seemed to be characteristic of the male cells. From the above

facts and the following evidence from its behavior, it seemed warranted to speak of this small chromosome as the Y and the other unpaired chromosome as the X.

From the late anaphase of the last spermatogonial division the nucleus reorganizes into a network with two nucleoli. The nuclear network becomes the large, flocculent, lightly staining masses commonly called prochromosomes, while the nucleoli remain distinct. As growth proceeds, these prochromosomes change into the spireme filaments. These autosomal threads present nothing irregular in their behavior. The two deep-staining nucleoli (fig. 2, A) unite so that in the early prophase of the first spermatocyte division only one irregular mass is seen. The actual union of these bodies takes place as early as the leptotene stage in some cells, while in others it is delayed until the diplotene.

Figure 2, D shows the ring type of chromosome formation in the prophase of the first spermatocyte division similar to that described by Payne ('14) in Forficula auricularia. In later prophase the chromatin nucleolus becomes distinctly bilobed and passes into the metaphase of the first division as an unequal pair of chromosomes. Figure 1, I shows several of these unequal pairs.

Figure 1, G and H show polar views of the first spermato-cyte division with 6 chromosomes. This number was constant in several hundred counts of the first and second divisions (fig. 1, G, H, K, and L). Figure 1, J shows all of the first spermatocyte chromosomes in side view. All the chromosomes including the unequal pair divide and appear as a dense clump of chromatin in late anaphase. There is a nuclear reorganization in interkinesis in which all chromosomes may be distinguished (fig. 2, F and G).

Following the resting stage, all six chromosomes divide in the second spermatocyte division, so that each spermatid receives 6 chromosomes (fig. 2, H).

From the above description it is evident that in Labidura bidens the diploid number of chromosomes is 12, or 10 autosomes and an XY pair. These chromosomes behave nor-

mally in both spermatocyte divisions so that each spermatid receives 6 chromosomes, i.e., 5 autosomes and either an X or a Y chromosome. From the study of the oogonial chromosomes, it is inferred that all the ova receive 5 autosomes and an X chromosome.

Labia minor Linn.

Material for the study of this species was collected at Winona Lake and Indianapolis, Indiana. Only a few specimens were obtained, but these were sufficient for working out all the meiotic stages except the late prophase of the first spermatocyte division.

In many ways the chromosomes of Labia minor resemble those of Labidura bidens. Even the form of the chromosomes is much alike in the two species (fig. 3, A, B).

The diploid number is 14. Figure 3, C shows the spermatogonial chromosomes grouped in homologous pairs with two chromosomes of unequal size designated X and Y. Only a few oogonial metaphase plates were found and none was very clear. Figure 3, D shows one of these with 14 chromosomes.

Following the last spermatogonial division, the nucleus reorganized into the network stage (fig. 3, E) where two deep-staining bodies were very conspicuous. This stage was followed by a period in which the chromatic material became massed into large irregular prochromosomes (fig. 3, F) which unravel to form the later spireme similar to that seen in Labidura bidens. The two deep-staining bodies of the earlier stage could be distinguished with difficulty among the large prochromosomes. As to their subsequent behavior, not enough material was available to trace them with certainty, but it is inferred that these two bodies unite to form the one deep-staining chromatin nucleolus in the later growth period (fig. 3, G), which, judging from the condition in other earwigs studied, probably becomes the unequal pair seen in the first spermatocyte metaphase.

From the numerous counts of first spermatocyte metaphase plates the number of chromosomes here was determined to be 7 (fig. 3, I, J). The side views of this stage showed an unequal pair to be present similar to that in Labidura bidens (fig. 3, H). Although it was not possible to compare the homologous pairs of the female with those of the male, the large chromosome of the unequal pair is probably the X element and the small chromosome the Y element (fig. 3, C).

The chromosomes separate normally in the first spermatocyte division and pass into the rest stage, where they remain as distinct masses. Figure 3, K and L give polar views of the second spermatocyte. Here the chromosomes divide normally passing into the spermatids.

Anisolabis annulipes Lucas

Specimens of this species were collected in the locality of New Orleans, Louisiana, Mississippi College, Mississippi, West Palm Beach, Florida, and LaJolla, California. By studying a great number of clear metaphase counts, the spermatogonial number in Anisolabis annulipes was shown to be 25 (fig. 4, A, B) and the female number 26 (fig. 4, C).

Following the last spermatogonial division and in the early growth period the nucleus contains two deep-staining bodies as in figure 4, D. (The plasmosomes have not been shown.) These bodies approach each other and unite (fig. 4, E), so that in the pachytene stage only one irregularly shaped mass is found. The actual time of union varies considerably in different cells, but it normally preceded the pachytene stage. As shown in figure 4, F, this deep-staining body becomes distinctly bilobed. In later prophases it condenses into a bivalent chromosome with one of the parts much larger than the other. Occasionally this chromosomal mass breaks up into a large bivalent and a small univalent, as in figure 4, H.

With the spermatogonial number of 25 it was to be expected that the first spermatocyte number would be 13, but such was not the case. Ninety-two per cent showed 12 chromosomes (fig. 5, A). The study of side views showed clearly

that a univalent chromosome did not lie in a different plane from that of the bivalent chromosomes, nor did it pass to the pole before the division of the bivalents. In a few cells examined a small univalent chromosome could be seen in the side views (fig. 5, C, D). Its presence plus the 12 bivalents accounts for the 13 chromosomes in some of the first spermatocytes (fig. 5, B).
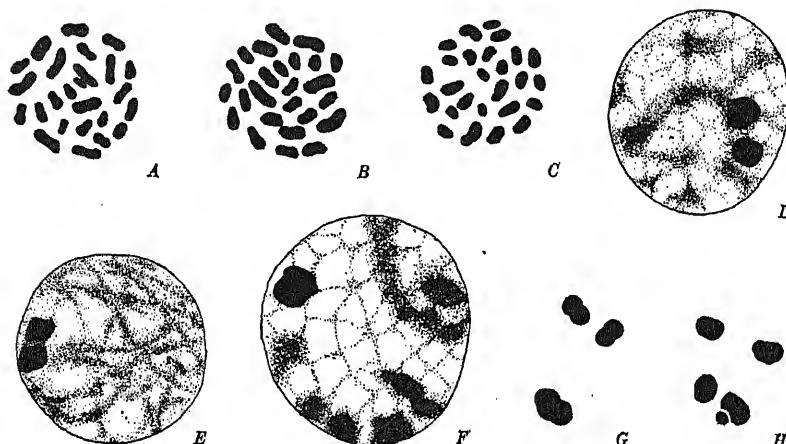
Fig. 4   A, B, spermatogonial metaphase, showing 25 chromosomes; C, oogonial metaphase with 26 chromosomes; D, E, F, growth period and early prophase, showing chromatin nucleoli becoming large bivalent; G, prophase chromosome group with large bivalent; H, prophase group, showing small element.

The metaphase plates of the second spermatocyte division showed no such irregularity in chromosome behavior as that found in the first division. Of the 513 counts made of the chromosomes of this division, 280 showed 12 (fig. 5, N) and 233 showed 13 (fig. 5, O). A study of the side views of metaphase and anaphase figures showed all chromosomes divided normally.

From the above facts the real problem in this species was to account for the disappearance of the univalent chromosome that was present in the spermatogonial metaphase and its reappearance in half of the second spermatocyte divisions.

. In the growth period and early prophase the dark-staining bodies gave rise to an 'unequal pair' of chromosomes which passed normally into the metaphase stage. A study of the side views showed only a few cases where a univalent chro-
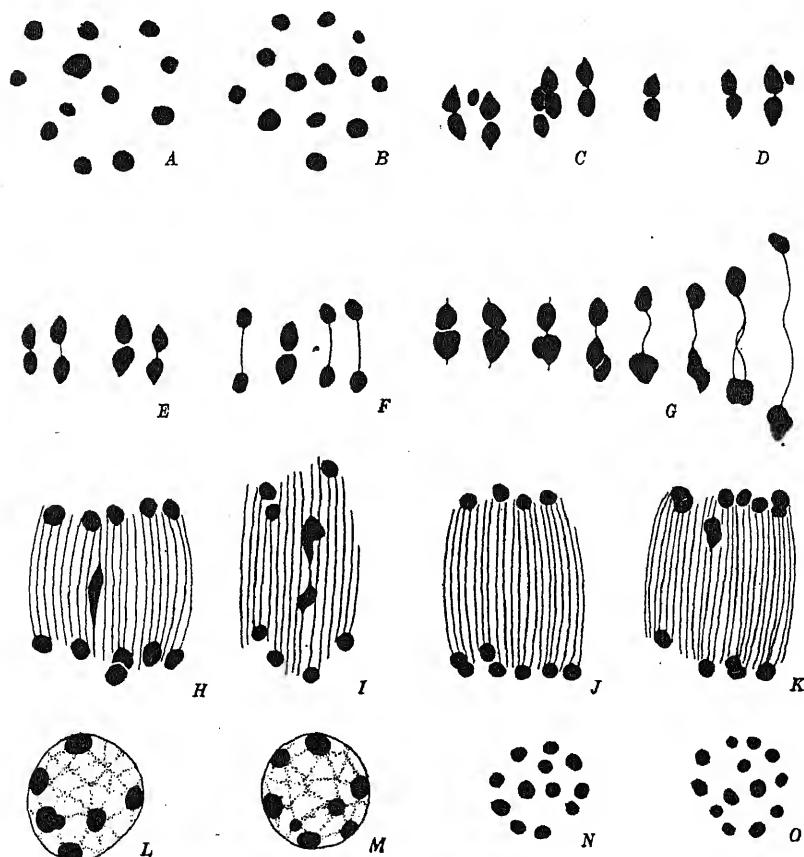
Fig. 5   A, first spermatocyte metaphase with 12 chromosomes; B, first spermatocyte groups with 13 chromosomes; C, D, side views, showing small univalent; E, F, side views, showing tripartite groups with parts nearly equal; G, tripartite groups in various stages of division; H, a typical lagging chromosome in first spermatocyte; I, division of lagging chromosomes; J, K, serial sections of a cell in later anaphase; L, M, resting nucleus between first and second spermatocyte divisions; N, second spermatocyte metaphase with 12 chromosomes; O, second spermatocyte with 13 chromosomes.

mosome was present. In these cells a close examination of the chromosome pairs showed that the parts of all bivalents were of equal size. This seemed to indicate that in the cells having an unequal pair of chromosomes the large portion of the unequal pair was in reality the univalent plus another chromosome. This 'unequal pair' will be referred to as the 'tripartite group' in later description. In a great many of these 'unequal pairs' the large part showed no indication of being duplex (fig. 5, E, F, and the first two groups in figure 5, G). As in figure 5, G, various forms in the large element of the tripartite group showed that it consisted of two components. Since one part of this tripartite group was in reality two chromosomes, the cell receiving this part would contain one more chromosome than its sister cell. The number of pairs being 11, each sister cell would receive 11 of these halves (diads) plus either the small or large part of the tripartite group. Figure 5, J and K are serial sections of the same cell in anaphase. By counting the chromosomes in both figures the lower part shows definitely 12 chromosomes, while in the upper part are 11 chromosomes and the bilobed mass that is slightly lagging. If this lagging individual is the larger part of the tripartite group representing two attached chromosomes, we have, then, 12 chromosomes passing to one pole and 13 to the other. In a study of the polar and side views of the late anaphase there was no evidence that these attached chromosomes separated before the resting stage between the first and second spermatocyte divisions.

Another point of interest in the study of the side views of the first spermatocyte division was the occasional lagging chromosome in the early anaphase stage. Although the number of lagging chromosomes in this species was very small when compared with Forficula auricularia, it was interesting because in Anisolabis annulipes only one of the chromosome pairs lagged behind the others during division. Figure 5, E and F show that the tripartite group lags slightly in the early anaphase. By following various stages of the division of

this group, it was found that many times the larger portion representing the two attached elements became very much elongated during the later anaphase. Figure 5, I shows the condition of lagging where the parts of the mass are dividing. One portion is very noticeably larger than the other. One suggestion to account for this condition is that the position of attachment of the univalent chromosome to the other member of the larger portion of the tripartite group brings about this lagging. When the univalent chromosome becomes attached so as to adhere to both members of the separating group the three chromosomes assume the linear arrangement in anaphase and later the whole mass is drawn out into the characteristic strand (fig. 5, H). Later anaphase showed these lagging chromosomes dividing unequally and passing into the condensed masses of late anaphase.

During the condensed condition of late anaphase, the individual chromosomes could not be traced. Following the reorganization of the nucleus in interkinesis, the chromosomes remain as distinct bodies distributed through the nucleus until the prophase of the second spermatocyte division. Part of the resting nuclei contained a chromosome smaller and denser than the rest. A further examination showed this small chromosome attached to another chromosome in many of the nuclei. It seems probable that this small individual is one of the double-X chromosomes which fused to form the larger portion of the tripartite group. Figure 5, L and M show this small chromosome which is probably separating from the large chromosome. The size difference of the elements which formed the larger portion of the tripartite group was not as pronounced as that shown in this figure. The difference here is probably due to the greater condensation of the small chromosome, because its outline is more definite and it stains deeper than the other chromosomes. Not all nuclei show the small chromosome, on account of its irregular distribution. It behaves as the other chromosomes in the second spermatocyte division, and the size difference was not as great as in the resting nucleus. It is probable that in

many cases the elements forming the larger portion of the tripartite group become detached as the individual chromosomes are separating from the dense anaphase clump during the reorganization of the nucleus.

From the above description it seems that the tripartite group of the first spermatocyte division is in reality a group of three chromosomes. Two of them, the X components, become more or less intimately attached during the growth period, and in most cases do not separate until during the reorganization of the nucleus prior to the second spermatocyte division. This accounts for the irregular number of chromosomes seen in the metaphase plates of the first spermatocyte divisions and the reappearance of the normal number of chromosomes in the second spermatocyte mitoses. The number of chromosomes passing to the spermatids was 12 and 13.

The behavior of the chromosomes during the meiotic divisions and their distribution to the spermatids have been traced, but there remains the interpretation of the chromosomes which went into the formation of the tripartite group (hexad). This chromosomal complex behaves in the growth period very much like the XY pair in Labidura bidens and Labia minor and is often very much like them in appearance (fig. 5, G). In Anisolabis annulipes the Y chromosome is represented as a single element, but the X chromosome of the other species is here represented by two components, that remain attached during the first spermatocyte division. A careful study of the side views of early anaphase, where the elements of the XX group may be distinguished by their lobing as in figure 5, G, indicates that the Y is slightly larger than either of the X chromosomes and that one of the X elements is slightly larger than the other. Since this size difference can be detected it makes it possible to determine that the 'univalent,' in the above description, does not become attached to *either* member of a pair of chromosomes, but to a definite one of the pair. It seems probable from the appearance of the 'unequal pairs' of the first spermatocyte that

one of the X components pairs with the Y chromosome and the other X component becomes attached to the first X. That this attachment is very intimate is shown by the appearance of the XX group in figure 5, E, F, and G. Since the large mass shows no break in its outline, one of the X components seems to be plastered to the side of the other. That the attachment of these chromosomes may be entirely broken in some instances is evidenced by the few cases of univalent chromosomes seen in the side views (fig. 5, C, D).

The question arises as to the origin of the double-X group. The intimacy of attachment of these two chromosomes in the first spermatocyte division suggests either of two conclusions: the two X chromosomes are in the process of uniting to form a large, single X chromosome as in Labidura bidens and Labia minor or in the process of becoming two independent chromosomes by splitting. In light of the observations on multiple X components made by other workers in insect spermatogenesis the second conclusion is the more tenable. Why these components remain attached during the first spermatocyte division and then act as independent chromosomes in the second spermatocyte division is not known. That the X components remain independent in the spermatogonial divisions is seen by the counts. Although the later behavior of the X components is very different in Anisolabis annulipes, their presence suggests a similar condition described by Payne ('09) in Conorhinus and Wilson ('09) in Syromastes.

Reviewing the chromosome distribution in this species, it has been shown that in the spermatogonial cell there are 22 autosomes and two X components and a Y chromosome. Prior to the first spermatocyte division, twelve pairs are formed. Eleven of these are autosomes and the other pair, whose parts are of unequal size, consist of two attached X components and a Y chromosome. At the first spermatocyte division eleven autosomes and the Y chromosome pass to one of the daughter cells, while the other receives eleven autosomes and the two X components. These divide regularly in the second spermatocyte. Since the female number is 26, it is

inferred that this number represents 22 autosomes and four X components.

Anisolabis maritima Bon.

The material for the study of this species was collected at Cold Spring Harbor, New York, and St. Petersburg, Florida. A few specimens were collected in other localities, but the bulk of the material came from St. Petersburg. Unfortunately, but few females were dissected and none of the slides from this material gave reliable counts.

Most of the slides for the study of Anisolabis maritima were prepared and the preliminary work completed before the publication of Kornhauser in 1921. In order to compare the spermatogenesis of this species with that of the other member of the genus, Anisolabis annulipes, the description is here given which, with minor exceptions, agrees with that of Kornhauser.

By counting the chromosomes of the clear metaphase plates of a great many individuals the spermatogonial number was shown to be 25 (fig. 6, A, B). A study of the side views of this division was made to make sure no chromosome or group of chromosomes were irregular in position. The chromosomes of both species of the genus Anisolabis are very similar in form and size so that the homologous pairs can only be determined by a careful study of exceptionally clear metaphase plates. Figure 6, C gives the results of an effort to group the homologous pairs, but the slight difference in the size and form of many of the chromosomes raises the question of accuracy of any such effort, although the grouping has been rechecked with many other clear cells. From the following description it will be shown that the chromosome distribution here is similar to that of Anisolabis annulipes, although the two species differ in many minor characteristics, so that they can be readily recognized from their cytological preparations. No effort was made to ascertain the chromosome number of the somatic cells.

After separating normally in the last spermatogonial division, the chromosomes lose their form and appear as lightly staining masses of granules in the typical network form of the resting nucleus. At this time one or more deep-staining nucleoli are to be seen. This network form of the nucleus seems to be merely a transition stage from the last spermatogonial division to the beginning of the growth period and is found in all the species of earwigs so far examined. In the
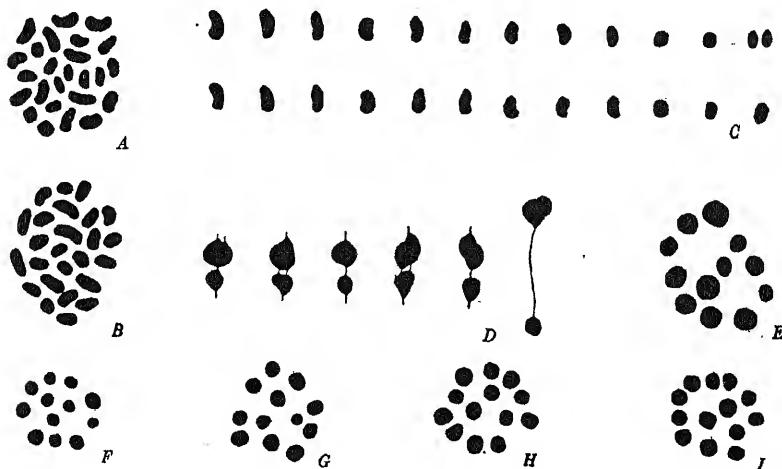
Fig. 6 A, B, spermatogonial metaphase plates with 25 chromosomes; C, homologous pairs of spermatogonial chromosomes; D, group of characteristic 'unequal pairs' of first spermatocyte; E, first spermatocyte metaphase with 12 chromosomes; F, G, second spermatocyte metaphase plates with 12 chromosomes; H, I, polar views of second spermatocyte metaphase with 13 chromosomes.

early growth period the chromatin becomes arranged in irregularly shaped masses or prochromosomes distributed through the nucleus. From the prochromosome stage the chromatic masses unravel to form the leptotene filaments. One or more plasmosomes are visible during the major portion of the growth period, but become smaller and disappear prior to the early prophase. The chromatin nucleoli seen in the short rest period may be distinguished with difficulty in the prochromosome stage, but later they are very prominent

in the nuclei, although their staining reactions are somewhat variable. During the growth period the two chromatin nucleoli become attached and appear as one deeply staining, irregular-shaped mass. The actual time at which this union takes place varies much in the different cysts and also in the different cells of the same cyst. The relative size of the fused nucleoli decreases as growth proceeds, probably due to condensation, and the outline becomes more nearly that of a bilobed body. In the prophase this bilobed condition is quite pronounced with one of the lobes larger than the other. At this time it is about the size of the larger autosome pairs. This deep-staining mass becomes a tripartite group similar to that described in Anisolabis annulipes. The above description of the formation of this 'unequal pair' and its subsequent behavior in later meiotic stages leaves little doubt as to its interpretation as an XXY complex.

By counting a great number of cells where there could be no possible question as to the number, it was found that in Anisolabis maritima there were 12 chromosomes in the first spermatocyte division (fig. 6, E). This species differs from the foregoing in that the 12 chromosome counts in this division were constant whereas in annulipes more than 10 per cent of the cells showed 13 chromosomes. A study of the side views showed one pair very similar in form to that of the tripartite group seen in annulipes. Figure 6, D shows the variation in form of the larger portion of the 'unequal pair.' Individuals regular in their outline were nearly as numerous as the lobed ones. In all probability, the large element is made up of two components as in the other species. The duplex nature of the large element can readily be distinguished either by the lobing or by a barely discernible division. In the first spermatocyte mitosis the autosomes and tripartite group divide regularly with none of the chromosomes lagging as in Anisolabis annulipes.

By counting many clear metaphase plates of the second spermatocyte division, it was found that the numbers of 12 (fig. 6, F, G) and 13 (fig. 6, H, I) were about equally distrib-

uted. The chromosomes divided regularly, as no lagging ones were seen.

Since the second spermatocyte contained only distinct, individual chromosomes, the large element of the first spermatocyte metaphase seemed to have been replaced by two smaller ones. The separation seems to take place during the reorganization of the nucleus. Occasionally, an interkinetic nucleus was seen in which two of its chromosomes were in contact. Probably this represents the two X components in which the separation had been delayed.

The large irregular bivalent of the late growth period of Anisolabis maritima is undoubtedly the homologue of the similar mass in Anisolabis annulipes. Since their origin and distribution are quite the same, the interpretation of the group of chromosomes given under the description of Anisolabis annulipes could be substituted here, i.e., an XXY-complex. This complex of three chromosomes (hexad) becomes arranged in the metaphase of the first division to appear as an unequal pair, since two of the three chromosomes remain in such intimate contact as to resemble a complete fusion.

Attention should again be called to figure 6, C, where an effort was made to arrange the spermatogonial chromosomes into homologous pairs. It was found that two small chromosomes were very nearly the same size, while the one chromosome without a mate was much larger. If no other evidence was at hand, it is probable the two small individuals would have been considered a pair of homologous autosomes with the unpaired individual, the X chromosome. However, the condition in the later meiotic stages indicates that this single individual is a Y chromosome which is limited to the male sex, while the pair of small chromosomes is in reality an XX group. These X components do not separate, but pass to one of the daughter cells in the first spermatocyte division. In the second spermatocyte they divide as independent chromosomes, so that each spermatid in such a mitosis received 11 autosomes and two X components. The spermatids result-

ing from the division of the cells containing the Y element receive 11 autosomes and the Y chromosome. From this distribution of chromosomes it is inferred that the female diploid number is 26, 22 of these being autosomes, while the other four are X components, two of which were derived from the 13 chromosome sperm.[2]

Forficula auricularia Linn.

All the material for the study of this species was collected in the locality of Zürich, Switzerland. The gonads were fixed in Flemming's fluid and, after sectioning, stained with Heidenhain's iron-hematoxylin. A great quantity of material was thus prepared which has permitted the study of all stages of spermatogenesis in many individuals.

Sinety, Stevens, and Meeks have stated in their studies that the spermatogonial or diploid number of chromosomes was 24. While these authors do not find variations, the work of Zweiger and Payne indicates that the spermatogonial number may vary from 24 to 26, with occasional 23 and 27 counts. With these conditions in mind, an effort was made to determine the true number or to find some key to the diversity of observations reported by former workers.

The chromosomes of the spermatogonial divisions are inclined to form into clumps which are difficult to count. For this reason counts were recorded only from metaphase plates where the chromosomes were far enough apart to make their individuality certain. By studying a great many individuals, it was found that the results would not justify the placing of any single number as characteristic of the species. About half the specimens studied showed 24 chromosomes (fig. 7, A, B) while the rest showed 25 (fig. 7, C, D). There were few counts of 23 and 26, but these have not been considered important, since there was doubt as to these numbers actually representing the number of chromosomes in the cells. The figures of the spermatogonial metaphase (fig. 7, A, B, C, D)

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[2] Kornhauser reports the female diploid number to be 26.

suggest how easily inaccuracies in counting might result in cells where two or more of the chromosomes were in contact in the metaphase plate. These conditions would tend to give counts with numbers lower than normal. The few plates with 26 chromosomes were in material that was poorly fixed, so that these counts were excluded from later considerations, since the chromosomes in other parts of the gonads behaved as in the individuals with either the 24 or 25 counts. Abnormal divisions with multipolar figures were not uncommon in the spermatogonia as well as the spermatocyte mitoses. After removing the specimens with uncertain counts, they were divided into two groups according to the number of spermatogonial chromosomes, one with 25 and the other with 24.
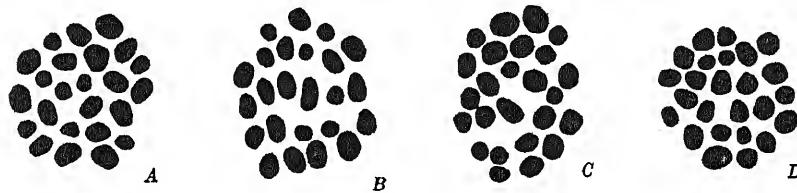
Fig. 7 A, B, polar views of spermatogonial metaphase with 24 chromosomes; C, D, same stage with 25 chromosomes.

Payne has described the formation of the first spermatocyte chromosomes. My findings are essentially the same as his. The conditions here were similar to those found in the two species of Anisolabis. The deep-staining nucleoli of the growth period gave rise to a bilobed body in the prophase (fig. 8, A) which became the unequal pair seen in the first spermatocyte side views.

More than 1300 clear metaphase plates were counted in a numerical study of the first spermatocyte division. Those individuals having 24 spermatogonial chromosomes showed regularly 12 chromosomes (fig. 8, K) while those with 25 spermatogonial counts showed three conditions. In these latter individuals one part had mainly 12 first spermatocyte chromosomes with very few 13 counts (fig. 8, J); the second group showed both 12 and 13 counts in about equal numbers,
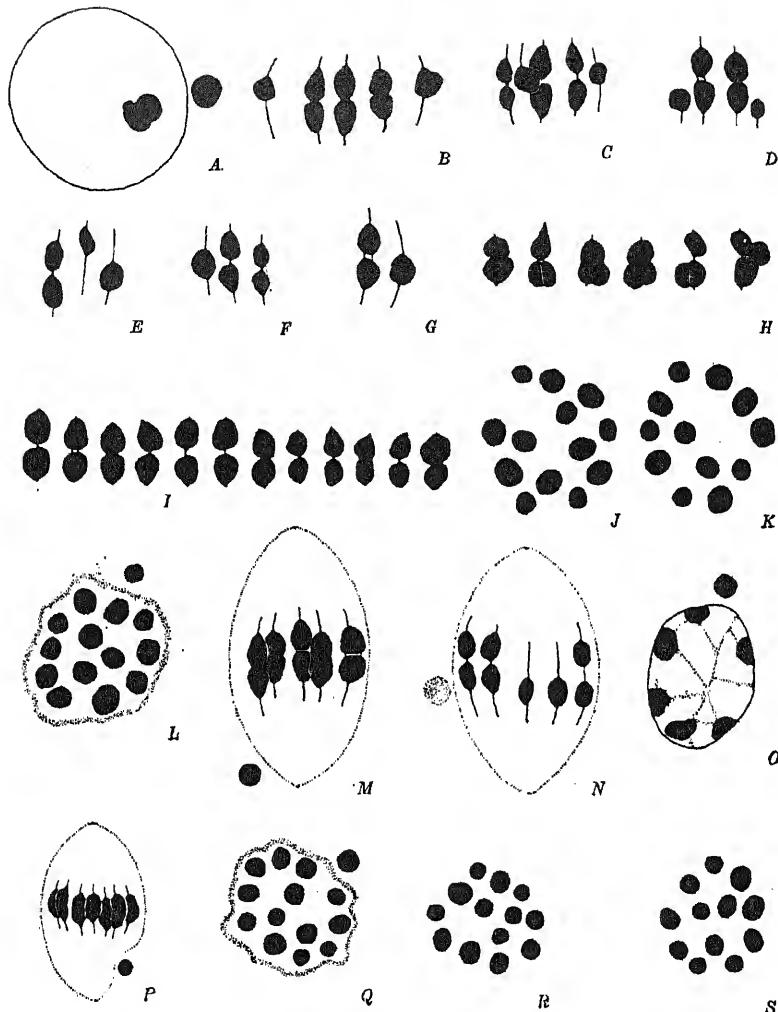
Fig. 8 A, cytoplasmic body near prophase nucleus, tripartite group only intranuclear structure shown; B, C, D, E, side views of first spermatocyte, showing two univalent chromosomes; F, G, single univalent chromosome in side views of first spermatocyte; H, tripartite groups of first spermatocyte; I, all the chromosome pairs of a single first spermatocyte cell; J, first spermatocyte metaphase with 13 chromosomes; K, same stage with 12 chromosomes; L, polar view of first spermatocyte, showing cytoplasmic body; M, N, cytoplasmic body in side views of first spermatocyte; O, resting nucleus between first and second spermatocyte divisions; P, side view of second spermatocyte; Q, polar view of second spermatocyte, showing cytoplasmic structure; R, 13 chromosomes of second spermatocyte metaphase; S, same stage with 12 chromosomes.

and in the third group nearly all the counts were 13 with only a few cells with 12 chromosomes. Very rarely was an 11-chromosome metaphase plate seen in this division.

Nearly 4000 counts were made of the clear second spermatocyte metaphase plates. After the counts in each specimen were tabulated, it was found that those individuals with 24 chromosomes in the spermatogonia and 12 in the first spermatocyte showed only 12 chromosomes (fig. 8, S), while those earwigs with 25 spermatogonial chromosomes and 12 or 13 first spermatocyte chromosomes had about equal numbers of 12 and 13 counts (fig. 8, R). A few specimens with 25 spermatogonial chromosomes showed most of the counts in both the first and second spermatocyte metaphase to be 13 with only a small percentage of metaphase plates with 12 chromosomes.

In 2017 counts of the spermatocyte divisions in the group of specimens with 24 spermatogonial chromosomes only thirty counts showed 11 chromosomes. All but six of these thirty counts were in the second division. In 1425 counts of the spermatocyte divisions of the individuals, with 25 spermatogonial chromosomes two counts in the first and eighteen in the second showed 11 chromosomes, while four counts in the first and twelve counts in the second showed 14 chromosomes. Since some of these were seen in cysts having cells with multipolar figures, they were thought to be due to some pathological condition and when these abnormal cysts were studied more closely, there was much evidence to indicate that whole cysts failed to complete the gametogenesis and form functional sperm. Many cysts with cells arrested in various stages of mitosis were apparently disintegrating low in the testis among masses of sperm. In several of these cysts there were clear eleven and fourteen counts, but since the condition was abnormal, no explanation is offered for their existence. The fourteen counts were present only in the individuals with the 25 spermatogonial chromosomes. There were a few cases of 11 and 14 spermatocyte counts in normal cysts that could not be explained on the above assumption.

It was to be expected that the distribution of the 25 spermatogonial chromosomes might be similar to that in Anisolabis, but this was only partly true, since there were usually 13 chromosomes in the first spermatocyte metaphase in these specimens of Forficula auricularia. In the study of the side views of this group, the presence of the univalent chromosomes at once attracted attention. With the 13 chromosomes in the first spermatocyte metaphase plates the condition could be interpreted as 12 pairs and one univalent. Figure 8, F and G show this condition, but another condition was also common, as indicated in figure 8, B, C, D, and E, in which there are two univalents, while a study of the other chromosomes showed that these were not the parts of a pair that had been displaced in sectioning. The size relation between these two univalents was similar to that existing between the parts of the tripartite group. In figure 8, B and C, the duplex nature of the larger univalent may be seen. This evidence seemed to indicate that the two univalents were in reality the parts of the tripartite group. The larger lobed 'univalent' being the two fused X elements, while the small univalent was the Y chromosome. Here the XX group had failed to pair with the Y chromosome or they had separated from the Y prior to the first spermatocyte metaphase. Figure 8, E indicates that these 'univalent' chromosomes pass to opposite poles, but figure 8, D and N suggest that they may both occasionally pass to the same pole. If this is ever the case, it may be one explanation of the occasional 11 and 14 counts seen in the second spermatocyte metaphase plates. The 14 counts might result if the large portion of the tripartite group separated into two single chromosomes, as it normally does in Anisolabis. From the great number of cells that disintegrate before their metamorphosis into sperm, it is reasonable to believe that these cells with the abnormal counts fail to become functional gametes. The two univalents passing to the opposite poles cannot be followed beyond the anaphase of the first spermatocyte division, because the chromosomes become arranged in a dense clump prior to their

interkinetic nuclear reorganization. Their behavior in the second spermatocyte division seemed to be similar to that of the parts of the tripartite group in Anisolabis, i.e., the smaller element behaves as the other chromosomes, but the larger element separates into two single bodies which later divided normally as individual chromosomes.

The single univalent is about the size of the smaller portion of the tripartite group. This indicates that the X components differ in size. In Anisolabis annulipes it has been shown that the tripartite group was an XXY complex, with the larger portion made up of two attached X components. In a small percentage of cases in A. annulipes it was found that one of these X elements separated from the complex, but passed to the same pole as the other X. This condition is similar to that found in Forficula auricularia.

Another item that has probably been a source of error in determining the chromosome counts in Forficula auricularia is the presence of one or more deeply staining bodies surrounded by a clear area in the cytoplasm. Figure 8, A shows one of these bodies in the cytoplasm very near the nucleus. Occasionally, one large body is present with a small mass lying near it, and at other times only small bodies are seen. These cytoplasmic structures are of interest here because they may easily be mistaken for chromosomes. Figure 8, L shows 14 bodies in a metaphase plate of the first spermatocyte division. All of these might have been considered as chromosomes, but, as indicated in the drawing one of them lies entirely outside of the division figure. Figure 8, Q is a similar case in the second spermatocyte division. In well-destained hematoxylin preparations these bodies begin to lose their stain before the chromosomes so that they appear in various shades of gray (fig. 8, N). In figure 8, O, one of these bodies lies in the cytoplasm in the resting stage between the first and second spermatocyte divisions, while figure 8, P is that of a second spermatocyte figure in side view showing one of these masses near one pole. In the figures only the larger bodies are shown. In many cells these

masses are so small as to be entirely overlooked, except in a study of cytoplasmic structures. It is not the purpose of this paper to deal with the cytoplasmic inclusions, but in this species these structures have added further confusion to a very variable chromosome behavior. It might be well to call attention to the fact that the tripartite group often lies at the periphery of the plate of chromosomes, so that its eccentric position might lead one to confuse this chromosomal mass with a cytoplasmic body, but a careful examination shows the cytoplasmic structures to lie outside the achromatic figure.

Payne and Stevens have called attention to the number of lagging chromosomes present in both spermatocyte divisions of Forficula auricularia. The number of these irregular chromosomes is very great in some specimens, so that some cysts in late anaphase, especially in the first spermatocyte, show most of the cells with one or more lagging bodies. In form these lagging chromosomes vary from short, rod-like masses to elongated cigar-shaped strands. A study of these lagging chromosomes showed that two were generally present and that one of these was either bilobed, i.e., two long rod-shaped masses attached end to end, or, if not lobed, was nearly twice the size of the others. Cells with three lagging chromosomes were also fairly numerous. Two of these masses were about the same size, while the third was larger. Several of Payne's figures show this condition. In most cases the bilobed portion or two individuals passed to the same cell, and the single portion, the larger mass in case of three, to the other. Some of these elongated masses failed to pass to either cell and seemed to hang suspended midway between the two chromosome clumps of late anaphase. Instead of these lagging chromosomes passing to the daughter cells, the other chromosomes which had divided normally receded, drawing nearer and nearer the median lagging bodies, until finally all appeared as one dark mass. At this stage the wall separating the two cells broke down, leaving the large clump of chromosomes in the center of a mass of cytoplasm twice the size

of a normal cell. The cell, resulting from this fusion then, seemed to undergo the usual reorganization following division. This gives a probable explanation of the presence of at least part of the many irregular cells with the double number of chromosomes. As to the interpretation of the characteristic lagging chromosomes of this species it seemed certain that they were the irregular members of the XXY group. The fact that one of these lagging masses was usually double, in size if not in outline, and that they passed to opposite poles was further evidence of their identity. The tripartite group of the first spermatocyte or its separated parts could not be distinguished in the cells in early anaphase with lagging chromosomes. Cases of one lagging mass that divided in very late anaphase were interpreted as a delayed separation of the Y from the X elements of the XXY complex. The above explanations cannot hold entirely for the lagging chromosomes of the second spermatocyte, but even here this irregularity was probably due to the abnormal behavior of one or more of the present sex chromosome group. The above explanation has not been offered to apply to those cells which seem to have lost all mitotic regularity due to some pathological condition. In some of these cells several lagging chromosomes were present, while other cells in the same portion of the cyst were in multipolar division. It was further observed that the lagging chromosomes were more often seen in specimens with the 25 spermatogonial number than in those with the 24 counts.

In a careful study of the side views of the first spermatocyte chromosomes of individuals with the 24 spermatogonial counts it was found that irregularities in the behavior of the chromosomes very seldom existed in this division. The tripartite group was usually similar to that shown in figure 8, I, but in some groups the larger part resembled that of the second individual in figure 8, H, where there is an indication of its being made up of two separate elements. With this last exception, the behavior of the XXY complex in this group of individuals was similar to the behavior of the unequal pair

in Labidura bidens, although in some of the tripartite groups in Forficula auricularia there was but little difference in the size of the two parts. The twelve pairs of chromosomes separated in the first spermatocyte division and these chromosomes behaved as single individuals in the second spermatocyte, since a 13 count was seldom seen.

In the group of individuals with the 24 spermatogonial chromosomes and 12 chromosomes in both the first and second spermatocyte divisions it seemed that the attached pair of X components retained their union throughout the maturation divisions. Whether or not this fusion was permanent has not been determined, but the behavior of this sex chromosome complex was so different from that in the group with 25 spermatogonial counts that these two groups of earwigs might be mistaken for two distinct species from a consideration of their cytological data. That there is no taxonomic evidence for such a subdivision of this species, Forficula auricularia, has been assured me by A. N. Caudell, of the National Museum, and James A. G. Rehn, of the Philadelphia Academy of Natural Sciences, who have gone over all my specimens with this point in mind. The variety forcipata Steph. seemed to be about evenly distributed between these two cytological groups. The explanation for the difference in the two groups seems to lie in the variability of the attraction of the X components for each other. In the group of specimens with 25 chromosomes this attraction may be so slight as to cause no attachment in either the first or the second spermatocyte divisions while in the group with the 24 chromosomes the attraction is so great that the two components remain attached and behave in mitosis as a single chromosome. The evidence indicated that in this species either a single X chromosome is in process of splitting to form two chromosomes or two X components are in the process of fusion to form a single large chromosome.

Only a few slides of female material gave clear-cut oogonial counts. Although the counts were too few to be used as evidence, it is of interest that these oogonial metaphase plates

contained 24 and 25 chromosomes. Payne has also figured an oogonial cell with 25 chromosomes. Oogonial counts with 26 chromosomes might also be expected. From the distribution described above, the spermatids in the 25-chromosome group, would receive 11 autosomes and either a Y chromosome or two separate X components, while the spermatids in the 24-chromosome group would receive 11 autosomes and a Y chromosome or a fused X group. If this fused chromosomal group remained in this condition, females of three oogonial counts would result. Some would have 24 chromosomes where both the egg and the sperm carried the fused X components; others would have 25 chromosomes where only one of the gametes carried the attached X components and in the other germ cell they were separate, and still other females would have 26 chromosomes, as in Anisolabis, where the X components in both gametes remained separate.

DISCUSSION OF VARIATIONS IN CHROMOSOME NUMBERS

Although the number of species used in this study is too small to warrant any broad generalizations to account for the difference in chromosome numbers found in the family Forficulidae, nevertheless, certain observed facts suggest an explanation of these numerical variations. Some of the questions which arise in this connection are: first, what outstanding condition is common to all species under observation? secondly, are the chromosome numbers of the species interrelated as to identity, polyploidy, or small numerical variations? thirdly, is there a great variation in chromosome size among the species? fourthly, which numerical condition is the most primitive, and is this correlated with taxonomic data?

Probably the most outstanding condition noted in the preceding descriptions was the presence of an XY complex. In both Labidura bidens and Labia minor this complex was represented by two distinct chromosomes which behaved entirely normal in all the meiotic divisions. In all five species the Y element behaved as a distinct chromosome. The X

element in Forficula auricularia showed two types of behavior, one with the X element as a distinct chromosome, the other with the X element divided into two separate components. In both species of the genus Anisolabis the X element was divided into two components, except for their adhesion during the first spermatocyte division. The behavior of the XY complex in Labidura bidens and Labia minor suggests a stable relationship existing between the X and Y chromosomes. In the other three species this complex shows an irregular behavior suggesting the probable fragmentation of a single X chromosome to form the two X components. This condition suggests the observations of Wilson ('09) and Payne ('09) on the multiple X element in the Hemiptera.

Among the five species of earwigs, three showed 25 to be the diploid chromosome number in the male. Although part of the individuals of F. auricularia showed only 24, this was interpreted as due to a fusion of the X components which remained separate in other members of this species. The number 12 found in Labidura bidens is but two less than that in Labia minor. In order to account for the origin of the chromosome numbers from a single type it would be necessary to assume that the autosomes have undergone two types of irregularities in their distribution, i.e., the loss or increase of single chromosomes (non-disjunction) and the doubling or halving of all or most of the autosomes (reduplication). Whether fragmentation or fusion has taken place can only be conjectured, although some evidence on this point may be gained by a comparison of chromosome sizes between the different species.

The chromosomes in all species were rod-shaped bodies of various lengths. In both species of Anisolabis the 25 chromosomes were short rods, slightly longer and more definitely separated in the metaphase plate than those of F. auricularia. Both Labidura bidens and Labia minor had two types of chromosomes, viz., very short rods similar to those in the other three species, and longer rod-like chromosomes much greater in length than those of the other species.

Some explanation for the variation in chromosome numbers among the five species may be found in a comparison of the size and form of the chromosomes of the two species having 12 and 14 with the three having 25. A comparison of the chromosomes in figure 3, C, with those in figure 1, F, indicates that both species have four pairs of chromosomes much larger than the other autosomes. Figure 6, C shows the chromosomes of Anisolabis maritima. Although the differences in the chromosome sizes are not as distinct as might be expected, it is at least interesting to note that, in general, the size relations suggest that the species with the low chromosome numbers may have arisen from a higher-numbered form by a fusion (chromosome linkage) of the chromatin bodies, or the species with the 25 chromosomes may have gained their higher numbers by fragmentation. The general tendency toward irregular distribution of the chromosomes, especially in the case of lagging, suggests one possible mechanism in bringing about changes in chromosome number. Whether any permanent changes are taking place at present is not known.

As to which condition, the low or the high chromosome number, is the more primitive has not been determined. It seems easiest to infer that 12 represents the most primitive number, and from these long chromosomes the higher numbers have arisen by fragmentation and reduplication. It may be noted in this connection that a rough estimate of the volume of the chromosomes of Labidura bidens approximates the volume of the chromosomes of the higher-numbered forms. Whether there is any correlation between the inferred primitive chromosomal numbers and the taxonomic position of the species is not known.

SUMMARY

1. The chromosomes of the five species of earwigs studied may be divided into, a) the autosomes and, b) the XY-complex.

2. The diploid number of chromosomes in Labidura bidens was 12 and in Labia minor 14; these consisted of 10 and 12

autosomes, respectively, plus single X and Y chromosomes. No irregularities were observed in the distribution or the behavior of the chromosomes of these two species.

3. In Anisolabis annulipes and Anisolabis maritima the diploid number was 25, or 22 autosomes and two XX components and one Y chromosome. The X components became attached to each other during the growth period and remained in this condition through the first spermatocyte division. The X components separated during the nuclear reorganization at interkinesis, and thereafter behaved as the other chromosomes during division.

4. The diploid number of chromosomes in Forficula auricularia was found to vary between 24 and 25, but the number was constant for the individual. Occasional spermatogonial counts were observed with numbers varying from those already stated, but these were attributed to poor fixation, the displacement of chromosomes in sectioning, or to pathological conditions.

5. In Forficula auricularia the number of autosomes was found to be 22 in all cases, but an irregularity was observed in the behavior of the sex-chromosome complex. In cells with 25 chromosomes there were two X components and one Y chromosome present similar to the two species of Anisolabis. In those specimens with the 24 chromosomes only a single X chromosome was visible with the Y chromosome. The observations indicated that this 'single' X chromosome was in reality two X components that remained attached during the maturation mitoses.

6. The variable results obtained by former workers on the chromosomes of Forficula auricularia were due largely to their failure to appreciate the behavior of the irregular XXY-complex. In some cases a cytoplasmic body was probably mistaken for a chromosome.

7. The observations indicated that the X chromosome is either undergoing a change from one large element into two small chromosomes, or two small chromosomes are becoming fused into a large single X chromosome.

8. The lagging chromosomes in Anisolabis annulipes and Forficula auricularia were interpreted as the irregular behavior of the XXY-complex.

9. There was no evidence indicating the chromosomal variations in Forficula auricularia were correlated with morphological variations of taxonomic importance.

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2 A, B, C, growth period, showing union of chromatin nuclei; D, E, single nuclei showing all six chromosomes; F, G, resting nuclei between first and second spermatocyte divisions; H, early spermatid.

3 A, B, spermatogonial group, showing 14 chromosomes; C, spermatogonial chromosomes grouped in homologous pairs; D, oogonial group with 14 chromosomes; E, nucleus following last spermatogonial division; F, early growth period, showing prochromosomes; G, later growth period with single chromatin nucleolus; H, unequal pairs from first spermatocyte, showing characteristic form; I, J, polar views of first spermatocyte metaphase; K, L, second spermatocyte polar views.

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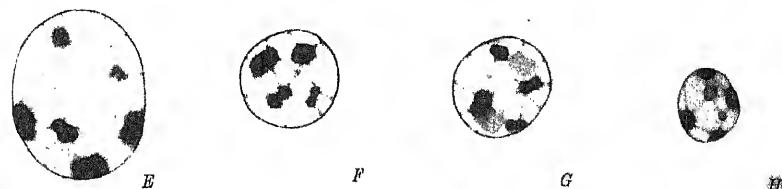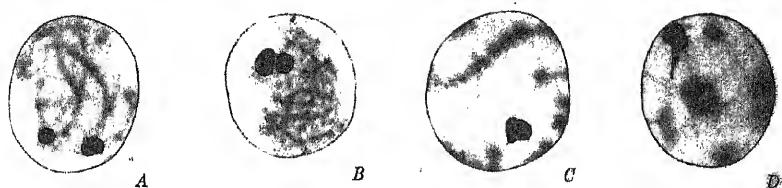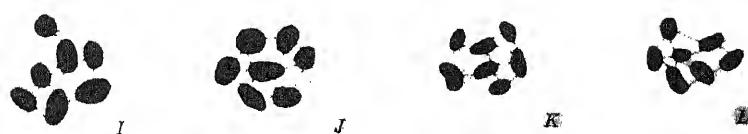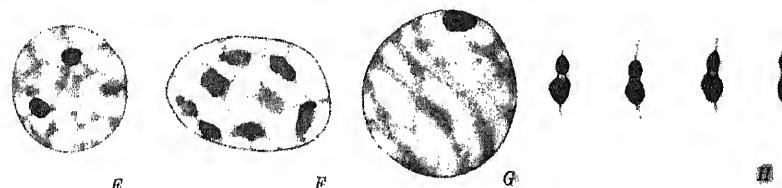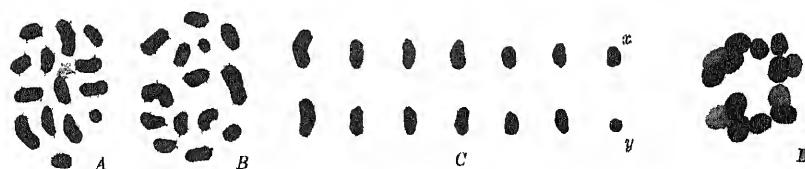
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THE HISTORY OF THE GERM CELLS IN THE DOMESTIC FOWL

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AUTHOR'S ABSTRACT

Material of the domestic fowl of appropriate ages, ranging from twelve hours' incubation to the adult bird, was prepared for the purpose of studying the production and development of the germ cells.

The primordial germ cells arise in the extra-embryonic region anterior to the head fold in the region of the zone of junction during the primitive-streak stage. These germ cells migrate, through the blood stream, to the region of the future gonad, where they develop into the definitive germ plasm.

There is no widespread degeneration of the primordial germ cells after their arrival in the gonadal region, nor is there any widespread transformation of somatic cells into definitive germ cells.

INTRODUCTION

Since the publication of the observations of Waldeyer, in 1880, on the origin of the germ cells in the embryo there have been many contributions to the knowledge of this subject. His views have been supported by many subsequent observations until at the present time many workers agree with him that the primordial germ cells arise from the germinal epithelium, which is a localized area of the peritoneum on the dorsal side of the coelom.

Nussbaum, however, in 1880, published a version of the history of the germ cells that has more recently become very widely accepted. He reached the conclusion that the germ cells are not derived from the germinal epithelium, but are segregated at a much earlier period in the formation of the embryo, and, indeed, he contends that their origin is traceable to the preceding germ cells. His views have been very stimulating to research on this problem, and it has received corroboration from so many investigators in all the vertebrate classes, with the exception of the mammals, that it may well be said to be the dominant idea of the present time.

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There are now two distinct schools of investigators, both of which have used the chick embryo as material for investigation and have obtained conflicting results from their studies. These two schools hold views which correspond roughly to those of Waldeyer and Nussbaum, and, in so far as the fowl is concerned, are represented by the work of Firket on the one hand and by that of Swift on the other.

Both schools agree on the earlier stages: that the primordial germ cells arise anterior and anterolateral to the embryo in a specialized region of the germ-wall entoderm, just at the margin of the area pelucida, during the primitive-streak stage and up to the formation of the third somite, and are found in the space between the entoderm and ectoderm. Later, the mesoderm arises, and these primordial germ cells, by amoeboid movement, pass into this mesoderm and into the blood vessels being formed there. They are carried by the flow of the blood stream into all parts of the embryo and vascular system. They remain distributed in this manner until the embryo reaches the 20–22-somite stage, at which time they become relatively more numerous in the germinal epithelium.

From this point the one group represented by Swift, working with the chick, contends that the primordial germ cells increase in number by division in the gonads until about the eleventh day in the female and the seventh day in the male, at which time they go into the quiescent stage of the normal resting germ cell. There is no degeneration of the primordial germ cells, nor is there any proliferation of the germinal epithelium to develop later into definitive germ cells.

The other investigators, of whom Firket is typical, hold to the view that the primordial germ cells, which arise in the extra-embryonic regions and migrate to the gonads, degenerate after they reach the gonads and that the definitive germ cells arise from a transformation of the germinal epithelium.

From a study of the results of the various workers in this field it can easily be seen that the history of the germ cell in many animals is not yet settled. From a comparison of

the plates of the various reports it would seem that the conclusion reached is not due to a difference in the animals studied, but more to a difference in the interpretation of the facts by the investigators themselves.

The present trend of investigations along this line is toward an extra-embryonic origin of the primordial germ cells and their subsequent development into the definitive germ plasm. The more weighty conclusions, as a whole, seem to substantiate the extra-embryonic-origin theory.

Because of these divergent views, it has seemed desirable to reinvestigate the history of the germ cell of the fowl. Therefore, the study of the problem was taken up at the suggestion of Dr. A. Richards, to whom the writer is greatly indebted for his valuable criticism and helpful suggestions throughout the course of this investigation. The writer wishes also to acknowledge his obligation to Mr. H. R. Hulpieu, who so kindly permitted a restudy of his material.

MATERIALS AND METHODS

The material used in this study consisted of thoroughbred Rhode Island Red and Leghorn eggs and chickens.

Samples were taken at desired intervals from twelve hours' incubation to the adult bird. The accompanying table shows the age of the samples. In the early embryonic life the samples were taken at one-half-day intervals. This close interval was desirable in order to be certain of the migration period and the early gonadal development. In the later embryonic life the samples were taken at a day interval, while after hatching the interval was extended to from eight to fifteen days.

Although the fixation used in previous studies of this problem appeared to be satisfactory for this particular work, it seemed desirable to obtain a fixation that would be of more cytological value and would possibly show more than the fixations previously used. Fowl tissue was accordingly fixed in the various standard cytological fixations, and the resulting slides were studied in an attempt to locate the undesirable

Summary of age, sex, and condition of the germ cells

Embryo

AGE	SEX	NO.	CONDITION
12 hours		3	Primordial germ cells in entoderm
16 hours		4	Primordial germ cells in space and in entoderm
24 hours		3	Primordial germ cells in blood vessels of splanchnia
46 hours		4	Primordial germ cells in entire blood-vascular system
55 hours		2	Primordial germ cells entering gonad region
3½ days		2	Primordial germ cells under germinal epithelium
4 days	Male	2	Primordial germ cells beneath germinal epithelium
5 days	Male	2	Germ cells in region of gonad formation of seminiferous cord
6 days	Female	1	Germ cells in germinal epithelial cords of first proliferation
7 days	Female	2	Germ cells in germinal epithelial cords of first proliferation
8 days	Male	2	Cords of first proliferation detached from germinal epithelium
9 days	Female	2	Germ cells in rapid division
11 days	Female	2	Germ cells in rapid division
12 days	Male	3	Growth of seminiferous cords by division of peritoneal cells
15 days	Male	2	Division of primordial germ cells sets in
15 days	Female	2	Germ cells entering first stage of maturation
16 days	Female	2	Continuation of maturation. Formation of synizesis
18 days	Female	2	Synizesis unraveling
18 days	Male	3	Rapid division of germ cells. Rearrangement of cords
20 days	Female	2	Appearance of leptotene threads

After hatching

AGE	SEX	NO.	CONDITION
2 days	Female	2	Rearrangement of follicle cells. Leptotene threads gone
2 days	Male	1	Appearance of lumen. No division of germ cells
10 days	Female	1	Accumulation of deutoplasm. Nucleus resting
12 days	Male	2	Syncytium appears. Lumen enlarged
35 days	Male	1	Enlargement of seminiferous tubule
37 days	Female	1	Condensation of follicle ring. Growth of ova
51 days	Male	1	Growth of organ
52 days	Female	1	Growth of ova
65 days	Female	1	Suggestion of renewed activity of nucleus
69 days	Female	1	Appearance of pachytene threads
70 days	Female	1	Pachytene stage
91 days	Female	1	Growth of ova. Nucleus resting
96 days	Male	1	Growth of organ
99 days	Female	1	Growth
115 days	Female	1	Growth of ova
126 days	Female	1	Growth
151 days	Male	1	Growth of organ
161 days	Female	1	Growth of ova
180 days	Female	1	Rapid accumulation of yolk material
Adult	Female	1	Oogenesis and ovulation
Adult	Male	1	Spermatogenesis

qualities and thus to work out a modified formula having the qualities necessary for the proper fixation of this material. After several attempts, a solution that seemed ideal was discovered and was used exclusively. It preserves the chromatin material of the somatic cells so well that an accurate count of the chromosome number is possible, while at the same time the grosser structures of the cells are well shown.

Since Woodgar ('25) attempted to trace the primordial germ cells by the cytoplasmic inclusions (Golgi bodies) and found that they did not differ materially from those of the somatic cells and that they could not be used as a definite criteria for the identification of the primordial germ cells, they were not taken into consideration while the study of fixation was being made. The important part of the cells considered in the formation of this fixation were those parts which could be used as definite criteria for the identification of the primordial germ cells, namely, attraction sphere, nucleus, cell walls, and chromosomes.

This fixation is composed of 15 parts chromic acid, 1 per cent: 4 parts potassium dichromate, 2 per cent, and 1 part glacial acetic acid. Material is fixed for twenty-four hours, dehydrated, and embedded in paraffin in the usual manner. Sections were cut at 4.5 and 7 µ. For staining purposes Heidenhain's iron haematoxylin was used exclusively.

CHARACTERISTICS OF THE PRIMORDIAL GERM CELLS

The most prominent criterion for the identification of the primordial germ cells is their large size. They are several times larger than the somatic cells, being from 10 to 15 µ in diameter. Swift ('14) states, "Isolated in the general mesenchyme they appear immense." They retain their large size throughout their development. Although they are slightly reduced in the later stages, they never become so small as to be confused with the somatic cells. The primordial germ cells are, as a rule, spherical, although there may be a few that will vary in shape until they will appear oval in sections.

The presence of a large attraction sphere to one side of
the nucleus that is found only in the germ cells is another
very good criterion to follow in their identification. Although
my material does not show the attraction sphere as prominent
as Swift figures it, it is nevertheless prominent enough for
use. The nucleus in most cases is placed to one side of the
cell and the attraction sphere lies in the space provided by
the eccentric placement of the nucleus.

The nucleus in most cases appears as large, if not larger,
than the entire somatic cell and is much clearer in staining
reaction than the somatic nucleus. The chromatin material
has a definite arrangement in the later stages, being gathered
into two distinct masses with a clear area between.

Yolk material is a very characteristic constituent of the
younger primordial germ cells. During the origin and migra-
tion of the germ cells they are simply overloaded with spheres
of yolk. As the embryo advances in age, the yolk material
within the germ cells decreases in amount, probably being
used as cell food. The yolk remains in the primordial germ
cells long after it has disappeared from the somatic cells.
This is probably due to the fact that, since the germ cells are
not going through division, they do not consume the stored
food as rapidly as does the dividing somatic cells.

The only sure way of identifying the primordial germ cells
is by using a combination of all the characteristics listed
above. By combining the large size, the nuclear condition,
the presence of the attraction sphere, and the yolk material,
the cell under consideration can in almost all cases be differ-
entiated as to whether it is a germ cell or a somatic cell.

METHODS OF ASCERTAINING SEX

The female differs from the male especially in the later
stages by the left gonad's being much larger than the right.
This size relationship cannot be relied upon much in the
earlier stages, for there is scarcely any difference in the size
of the two organs, although it is the only indication necessary
in the later stages.

The thickening of the germinal epithelium of the female gonad in the earlier stages is also an important character. The germinal epithelium of the male is composed of only one layer of epithelial cells, while in the female it is composed of a thick layer of cells which, especially in the left gonad, contains germ cells. This thickening of the germinal epithelium in the female is thought by Swift to be the most valuable criterion for sex determination.

The criteria of relative size of the right and left gonads and the thickness of the germinal epithelium permit the identification of sex at about the sixth day of incubation, while, by using the chromosome complex, sex can be distinguished at any age. Whether the peculiar-shaped chromosome really controls sex in the developing embryo or is merely characteristic of the sex is immaterial in this phase of study, for the fact remains that the chromosome complex of the male fowl contains two V-shaped chromosomes and that of the female, only one. Since these V-shaped chromosomes are always constant in the two sexes, they give a more trustworthy criterion for sex determination than any other known characteristic. Material fixed in the solution described above shows the chromosome complex clearly.

ORIGIN, MIGRATION, AND THE INDIFFERENT STAGE

The primordial germ cells are first to be seen in the embryo during the primitive-streak stage. They are to be found at the outer edge of the proamnion, just at the margin of the zone of junction, anterior and anterolateral to the head fold (fig. 1). They bud off from the inner portion of the entoderm and are found in the proamnionic area within the space between the entoderm and ectoderm before the mesoderm arises (fig. 2).

Later, when the mesoderm extends into this area, the primordial germ cells become incorporated into it and pass, by amoeboid movement, into the blood vessels which are being formed there. They follow the flow of the blood stream in the extra-embryonic region and up to about thirty-three-hour

incubation may be found only in the extra-embryonic regions (fig. 3). No primordial germ cells can be found in the embryo proper during this period, although they are relatively common in the extra-embryonic region.

Dantschakoff ('08), while studying the development of the blood cells of the fowl, observed that certain large cells were budded off from the entodermal germ wall anterior to the primitive streak, to which she gave the name, 'entodermal wandering cells.' These cells penetrate the walls of the blood vessels and are carried around in the blood stream. She reports their disappearance from the blood stream at about the time of the formation of the 21st somite. She described these cells as being larger than the blood cells, with many yolk spheres, and often showing pseudopods. Swift ('14) found these entodermal wandering cells to be the primordial germ cells in migration. The material used in this study shows very clearly the same condition as described by Dantschakoff and Swift.

At about thirty-three hours' incubation, the embryonic and extra-embryonic circulatory systems anastomose, and following this age the primordial germ cells can be found in the embryo proper.

Since no primordial germ cells are found in the embryo until the junction of the two blood systems, it is to be assumed that the only point of origin is outside the embryo and that the germ cells depend upon the blood system to take them to the regions of the future gonads (figs. 3 and 4).

The primordial germ cells remain distributed throughout the embryo until about the fortieth hour of incubation, at which time they become relatively more numerous in the region where the future gonads are to develop. They are still common in the blood stream, and some are to be found in such out-of-the-way places as in the mesoderm of the head (fig. 5) and in the splanchnic entoderm. These germ cells which fail to reach the gonads probably degenerate where they are.

In the two-day embryo the majority of the primordial germ cells are to be found gathering in the splanchnic mesoderm,

although some still remain in the blood stream. Those which still remain in the blood vessels are, as a general rule, near the curve of the coelomic cavity closely related with the region of the radix mesenterii.

In the three-and-one-half-day chick the primordial germ cells are located in the radix mesenterii and coelomic epithelium on both sides of the coelomic angle.

In the four-day embryo the germinal epithelium is developed along the median surface of the wolffian body. It is composed of cuboidal and columnar cells. Along the anterior portion of the wolffian body the gonads are being formed by the appearance of a stroma beneath the germinal epithelium. Located throughout this stroma and in the adjacent region are the primordial germ cells. However, by far the greater number of these cells are located in the gonad area proper (fig. 7).

In the stages when the primordial germ cells are in the blood stream and also from the time of leaving the blood stream until they reach their final destiny in the gonads they are at most times distinctly amoeboid. Although they are capable of throwing out pseudopoda while in the blood stream, these processes are not as definite or elongated as they become during the period of active migration from the blood stream to the gonadal region.

THE FEMALE

Embryo

In the five-, six-, and seven-day embryos the sex can be fairly accurately ascertained by the relative size of the right and left gonads, and by the thickness of the germinal epithelium, although, to be positive of the identification, it is well to rely upon the chromosome complex. Prior to this age, the development is practically the same in both sexes, and since previous workers have placed these ages under the indifferent stages they have been discussed in this paper under the same heading. If we accept the chromosome theory of sex in-

heritance, these earlier stages are not indifferent to sex, but are merely parallel in development, the differentiating marks not becoming evident until the end of this period, but the sex of any stage can easily be determined by a study of the chromosomes.

During the fifth day of incubation, the cords of the first proliferations are beginning to develop. The thickness of the germinal epithelium remains about the same, but along the inner surface there are budding processes to be seen growing out into the stroma. The buds are the first appearance of the cords of the first proliferation. Although the primordial germ cells do not take an active part in this growth, they are carried along in these budding processes. After the cords are extended to a considerable depth into the underlying stroma, they become separated from the germinal epithelium. These cords increase to a considerable size before they become broken up to form the greater portion of the tissue mass beneath the epithelium. Although they become broken up and fragmentary, they still retain their original orientation perpendicular to the germinal epithelium.

During the sixth day of incubation, the formation of the cords of the first proliferation ceases rather abruptly, and it is at this time the sex of the embryo can be definitely established by the use of the old criteria.

During these ages (five, six, seven days) there is very little division among the primordial germ cells. From the time they reach their position in the gonads to about the eighth day of incubation they remain in a quiescent condition (fig. 9), resulting in the necessity of using the somatic cells when a study of the chromosome complex was made for the identification of sex.

The eight-, nine-, ten-, and eleven-day embryos can be classed together, since the main activity of the ovary of these ages is the rapidity of division which occurs in the primordial germ cells during the formation of the cords of the second proliferation (fig. 10). These cords are composed almost entirely of primordial germ cells.

Swift ('15) reports a rearrangement of the mitochondria of these ages into the mitochondrial crescent, and he believes this rearrangement to mark the end of the primordial germ cells and the beginning of the definitive germ cells, the oogonial and spermatogonial generation. As stated above, the fixation used in this study was not prepared with the idea of preserving the cytoplasmic inclusions, so this material does not demonstrate the mitochondrial crescent described by Swift.

The ovary of the eleven-day embryo is becoming large, due, in a great measure, to the growth of the cords of the second proliferation and the accompanying increase in the number of the primordial germ cells (fig. 11). The medullary cords in the portion of the gonads toward the wolffian body have become longer and thicker with cavities developed. These cavities are lined with epithelial cells similar to a lumen and radiate toward the germinal epithelium.

The next stage worthy of note in the female germ plasm is the fifteen-day embryo. There are three very distinct changes in the primordial germ cells in the cortical region of this age: First, the cells are somewhat reduced in size, but they are still much larger than the epithelial cells. Secondly, the nuclear wall is broken down, and, thirdly, the chromatin material shows a decided tendency toward a massing (fig. 13).

Probably the best criterion for the identification of the primordial germ cells of this and the subsequent stages is the nuclear activity. Firket ('14) reports that it is at about this age that the degeneration of the primordial germ cells sets in; and, indeed, those cells within the region of the cortical cords of this stage do strongly indicate such a beginning of degeneration.

As far as observed, there was very little indication of cell division in the cortical cords of this age. All the primordial germ cells are going through this same change in appearance. The nucleus loses its clear staining reaction and many chromatin clumps are scattered throughout the karyo-

plasm. In some cells a portion of these masses are connected by thin strands of chromatin, while in other cells the masses are lying free with no connections between each other.

The above changes, with several important additions, are also to be seen in the sixteen-day embryo. The small chromatin clumps described in the fifteen-day embryo have become, in nearly all cases, aggregated into one irregular mass. The cytoplasm is clumpy and irregularly distributed and the karyoplasm shows practically no staining reactions (figs. 14 and 15). The primordial germ cells of this stage certainly suggest degeneration to a marked degree, and if one were observing only the fifteen- and sixteen-day embryos there would be no doubt in his mind as to the occurrence of degeneration. However, the later stages show conclusively that this is not a degenerating process.

In the eighteen-day embryo, contrary to expectations, the irregular masses of chromatin described in the sixteen-day stage are beginning to show signs of a loosening up or unraveling instead of further indications of degeneration (fig. 16). The nucleus of this age resembles very much that of the fifteen-day stage. The chromatin masses are again distributed in smaller clumps with connecting strands of chromatin between. However, the strands of connecting chromatin in the eighteen-day ovary are decidedly more definite and condensed in character than those strands of the fifteen-day stage.

The cytoplasm is losing its irregular appearance and is returning to a more healthy and normal appearance after the degenerative condition during the fifteen- and sixteen-day stages. The karyoplasm is again normal in staining reaction and does not show the sickly appearance as described for the sixteen-day ovary.

It is very evident that the fifteen-, sixteen-, seventeen-, and eighteen-day stages above described are normal synizeses in which the prochromosomes have migrated to a common point and have become massed together into a more or less dense intensely staining knot. Such an appearance of synizesis before the occurrence of the leptotene threads have been

demonstrated by Nonidez ('20) in the beetle Blaps, and by Arnold ('08) in Hydrophilus.

The epithelial cells throughout the ovary and the primordial germ cells outside the cortical region appear as normal resting cells in every respect during this time. Mitotic figures are very rare during this time, due probably to the fact that the strength of the organ is being used up by the primordial germ cells in their transformation.

By the time the embryo reaches the age of twenty days' incubation the chromatin clumps described as beginning to unravel in the eighteen-day stage have become beautifully spun out into definite leptotene threads of the bouquet stage with a basal chromatin mass from which the threads radiate (fig. 17). The cells are quite a bit larger than those in the earlier stages of synizesis.

As is true in any animal, the earlier stages of synizesis here suggest degeneration to a great extent, and unless the later stages, in which the leptotene threads appear, are clearly fixed and stained, the conclusion that degeneration does take place is excusable.

During the study of all of these stages there has been no hint of a transformation of epithelial cells into germ cells observed. The epithelial cells in all ages are of the same general size and in the same general condition, such as would be expected of any definite somatic tissue.

The study made on this material shows conclusively that the conditions simulating degeneration reported by Firket ('14) to begin at about fourteen or fifteen days' incubation and to continue through to about fifteen days after hatching are not degenerative processes, but rather are only the normal synaptic stages found in the oogonia and spermatogonia of many animals. Although synapsis has not been previously reported for birds, these stages just described are clearly synapses and conform definitely to the synaptic stages which occur in other forms.

It seems very probable that these stages were overlooked by Firket or that his material did not show well-fixed cases

in which these stages could be satisfactorily studied. This material has been shown to numerous students of cellular phenomena, and the interpretation here given has received full credence from them.

<div style="text-align:center">AFTER HATCHING</div>

In the two-day chick the leptotene threads are still distinguishable. They are not as clear as they were in the twenty-day embryo, and the basal chromatin mass has disappeared. The nuclear walls are still absent, although there is a slight indication of its reappearance. The follicle cells derived from the epithelium are beginning to be placed around the definitive ovum as the first indication of the formation of the follicle ring (fig. 18). We shall see in the later stages that these cells do form the follicle, as is indicated in this growth.

Although Swift takes the appearance of the mitochondrial crescent to indicate the transformation of the primordial germ cells into the definitive ova, it seems more plausible to consider the first appearance of the synizesis stage to be indicative of the transformation of the primordial germ cells into definitive ova. This, then, would cause us to consider the germ cells of the fifteen-day embryo to be the definitive ova.

In the ten-day chick the primordial germ cells or definitive ova, as they should now be called, have returned to the typical resting stage. The leptotene threads have all disappeared and the nuclear wall is again evident. The nucleus itself is in or near the center of the cell and is in a reticulated condition, showing no signs of further activity. The cells have attained a size considerably larger than the original primordial germ cell, due to placement of yolk material or deutoplasm. They stain very lightly and are very numerous in the cortical region of the ovary. They are now very definitely surrounded by the follicle ring (fig. 19).

Not all of the germ cells enter into this growth period, but relatively only a few. It may be that those cells which are

destined to be extruded during the first ovulation period are the ones which exhibit this growth. The follicle ring is not yet compact nor arranged in the condensed ring of the later stages.

In the thirty-seven-day chick the definitive ova have increased in size to a marked degree. The amount of deutoplasm has increased to such an extent that the follicle cells are forced out into a densely staining ring. The cells of the follicle are still cuboidal in shape, but instead of the longitudinal axis of the cell being parallel to the egg nucleus, they are now placed at right angles to it. The nucleus has not as yet started its migration to the periphery of the ovum, being still at or near the center of the deutoplasm. The deutoplasm shows no differentiation into different types of granules, being a continuous mass of homogeneous material.

In the forty-six- and fifty-two-day chick the border of large yolk granules is noticeable around the outer edge of the cell. This is the first appearance of the differentiation of the yolk material into large and small granules so noticeable in the later stages. The large granules are placed in a matrix of the smaller granules that was present in the earlier stages. There is a definite gradation in the size of the yolk spheres with the larger ones near the periphery and the smaller ones toward the nucleus (fig. 21).

In the sixty-five-day chick the nucleus shows signs of renewed activity. Darkly staining bead-like masses of chromatin material are arranged in linear rows with indications of attachment between each mass. Although these rows are very short and irregularly arranged, they strongly suggest a reappearance of the chromatin threads which were disintegrating in the two-day chick (fig. 20).

In the sixty-nine-day chick the nucleus has become temporarily definitely activated, and the threads that disappeared just after hatching are again evident. These threads are much heavier and thicker than the former threads, and the number (eighteen) corresponds to the haploid number of chromosomes (fig. 22). The haploid number and the heavier

appearance of the threads indicate the pachytene stage rather than the leptotene. Since it is reported that the ova do not enter into the maturation divisions until after the breaking of the follicles, these stages probably represent only the pachytene. The nuclear wall again breaks down during this stage. The oogonial nucleus returns to the typical resting stage following this activity, and the growth of the egg continues.

In the ninety-one-day chick the nucleus of the developing ovum has returned to the normal resting stage, with no signs of further activity. It remains in the center of the deuto-plasm. The follicle and deutoplasm are in the same general condition as described for the earlier stages, with the only noticeable difference being in the increase in size (fig. 23).

From the ninety-ninth day to about five and one-half months after hatching, the ovum continues its steady increase in size, with no outstanding changes, except that of growth. The large and small granules of yolk material are still discernible, the larger ones being placed near the periphery of the egg (figs. 24, 25, 26).

Just before the chick reaches sexual maturity the growth rate increases very much, resulting in the final supply of deutoplasm being collected very rapidly. At about six months after hatching, the ovum has increased to such a size that it is now supported from the ovary by the follicle stalk. It is during this last period of growth that the nucleus migrates to the periphery of the egg, causing the appearance of the nucleus and neck of Pander in the ovulated egg.

The foregoing description includes only the oldest of the developing ova in each stage. In all stages every size of ova may be found, from the resting primordial germ cells to the larger ones described.

Because of the large size of the developing ova, it appears that practically the entire ovary is going through this growth period, but this is due to the increase in the size of the definitive ova, and not to any decrease in number of the germ cells.

THE MALE

Embryo

The male embryo of six, seven, eight, and nine days' incubation (fig. 28) can be grouped together under the same discussion, since there is no abrupt changes occurring in these ages. It is during this period that sex is easily ascertained by the relative size of the right and left gonads, the thickness of the germinal epithelium, and by the lack of the cords of the second proliferation in the male.

The cords of the first proliferation appear during the sixth day of incubation, as was observed in the female and, like the female, the primordial germ cells take no part in this formation. These cords are first noticeable as small projections from the germinal epithelium growing down into the underlying stroma. Although they are not entirely straight in line of growth, they all radiate toward a common point—the place of attachment of the gonads to the wolffian body.

The entire gonad is changing shape during this stage. It is becoming thicker and shorter and is gradually increasing in size, while the attachment to the wolffian body is not so pronounced as in the younger ages of this same stage.

The cords of the first proliferation which originate as outgrowths of the germinal epithelium are to form the seminiferous cords of the older stages and are the part of the gonad in which we are most interested. During this stage, these cords are separated by a very thin layer of stroma. They are composed of two kinds of cells: the peritoneal cells which are derivatives of the germinal epithelium and the primordial germ cells which are carried in by the epithelial outgrowth.

The peritoneal cells are much more numerous than the primordial germ cells and still retain their cylindrical form. The germ cells are as outstanding in this stage as they were in the preceding stages and, with the exception of a slight reduction in size and the absence of vitellus, are unchanged.

In the later ages of this stage the stroma is developed more just under the germinal epithelium than in the other parts of

the gonad, thus forming the albuginea, which is a layer of tissue between the germinal epithelium and the seminiferous cords. In the remainder of the gonad the stroma still show the typical mesenchymal syncytium of the earlier stages.

In the ten-, eleven-, and twelve-day embryo the seminiferous cords do not extend in the regular manner as previously described, but form a sort of network with extensions in all directions. The cords have grown in length to a great extent, thus causing foldings and convolutions. The peritoneal cells are the principal ones present in the cords of this stage, although several primordial germ cells are to be seen in a single field (fig. 29). These germ cells are still in a quiescent condition. A new type of structure, the densely staining interstitial cells which appear in small groups in the stroma, is noticeable in this stage.

The testes have enlarged to a considerable extent and are more rounded in shape. The albuginea is much thicker than in the preceding stage and the stroma between the cords is gradually increasing in amount.

In the thirteen- and fourteen-day embryo the testes are still larger than in the preceding stage, due to the great increase in the amount of stroma. The organ is attached to the wolffian body by only a very thin layer of connective tissue. The layers of stroma between the cords are almost as thick as the cords themselves at this age.

The seminiferous cords now form an open network after having anastomosed with each other at every conceivable angle. They are larger in diameter than before, due to the rapid increase in number of the primordial germ cells. The division rate of the peritoneal cells seems to decrease as that of the germ cells increases. The cords stain less intensely than before, so that they stand out clearly even under the low-power lens.

From fifteen to eighteen days' incubation the most outstanding characteristic of the testes is the great amount of division of the primordial germ cells. In so far as the increase in number of germ cells is concerned, this stage is comparable

to the eight- to eighteen-day female embryo. The seminiferous cords branch and anastomose to form a network, as described earlier; however, they contain more germ cells than they did in the previous stages. The relative amount of cord tissue as compared to the stroma has increased considerably, due, of course, to the fact that this is the period of great increase in number of the primordial germ cells (fig. 30). Divisions among the peritoneal cells is at a standstill, although there is no sign of degeneration of these cells. The interstitial cells which are probably formed by transformation from the stroma cells are more numerous than ever before.

In the stage of eighteen days' incubation to hatching, the seminiferous cords are very large as compared with the previous stages (fig. 32). The interstitial cells, which were so numerous throughout the stroma of the preceding stages, are disappearing. The greatest change is taking place within the seminiferous cords. The primordial germ cells or spermatogonia, which have been scattered throughout the cords, are now moving or being moved toward the basement membrane of the cords. Swift's ('16) suggestion, that they may have regained the amoeboid power of the ancestral primordial germ cells, is very plausible, since many cells with pseudopoda extended toward the membrane are to be seen.

It is during this stage that the lumen appears as a cleft in the center of the seminiferous cords. The network of the cords are becoming reoriented, so that they extend from the germinal epithelium toward the wolffian body. The peritoneal cells are also arranged along the basement membrane of the cords, due, not to any amoeboid power of these cells, but rather to the fact that the center of the cords are being liquefied during the process of lumen construction, leaving intact only those cells near the membrane.

After hatching

In the two-day chick the seminiferous cords practically fill the entire testes and the stroma is relatively much reduced.

The cords now show a distinct though small lumen and should be spoken of as the seminiferous tubules. The peritoneal cells are elongated, so that they resemble somewhat the supporting cells of the adult organ, while the spermatogonial cells show but very little mitotic activity (fig. 33).

Swift ('16) reports that when mitosis does occur, the figure is so arranged that the line of cleavage is at right angle to the longitudinal axis of the tubule. In the material used in this study mitotic figures are arranged at all angles to the basement membrane, although the majority are placed as described by Swift.

In the twelve-day chick there is no great change to be seen above the two-day stage. The lumen is gradually increasing in size, although it is still merely a small tube-like opening, and not the great tube figured by Semon ('87). The reduction in the amount of stroma and interstitial cells is still going on, so that very few interstitial cells remain and the amount of stroma is noticeably reduced. The cell walls of the spermatogonia appear to be breaking up and a syncytium is being formed (fig. 34).

From this stage on in the development of the testes there are no sudden outstanding changes to be seen. It is more of a slow continuous process which goes forward gradually until maturity is reached. The slit-like lumen of the twelve-day chick slowly becomes larger as the process of liquefaction continues, until finally the solid seminiferous cords of the embryo become the large hollow tubule of the adult.

When the chick reaches maturity, the spermatogonial cells pass through the synaptic stages shown in the late female embryo, the two spermatogonial divisions take place and the spermatozoon is formed (figs. 35 to 37).

SUMMARY AND DISCUSSION

This study has corroborated the work of Swift in that no widespread degeneration of primordial germ cells has been found. Following the period of migration, the primordial germ cells come to rest in the gonads and, in the female em-

bryo of eight to eleven days' incubation and the male of fifteen to eighteen days' incubation, pass through a period of rapid increase in number by division, during which time the gonads increase rapidly in size.

Following this period of increased mitotic activity, the female germ cells enter into the first stages of synapsis at about fifteen days' incubation and continue through the synaptic stages until two days after hatching. At two days after hatching, the definitive ova begin to fill with deutoplasm, and this process continues until the egg is mature and ovulation takes place.

This study has not demonstrated that no degeneration of the primordial germ cells takes place. Such cells simply have not been seen. It has certainly demonstrated that degeneration is not the usual process and that if it does occur in some cases, enough primordial germ cells are left behind to account for the definitive germ cells without calling in the germinal epithelium.

A considerable part of the interest in this problem is traceable to the germ-plasm theory of Weismann, that the multicellular organism consists of two distinct parts, the soma and the germ plasm. As the germ-plasm doctrine was formally interpreted, it and the views of Waldeyer and the school to which he belongs, that already differentiated soma tissue could give rise to germ cells, are certainly not in accord. Perhaps this fact has been largely responsible for the great amount of interest in this problem.

Recent studies have brought about a reinterpretation of Weismann's hypothesis, and it is now held that the chromatin material represents the germ plasm, while the cytoplasm is the somatic material. Those cells in which the cytoplasm is especially differentiated becoming the somatic cells, while those which retain their general embryonic character furnish the germ cells.

It is evident from this interpretation that the problem of the origin of the germ cells become of interest from an embryological rather than a genetical point of view. It may be

said, however, that the work here reported offers no evidence that the germ cells are not segregated at an early period in the development of the chick embryo nor that there may not be a quite definitely maintained continuity of the germ plasm even in the earlier sense of the doctrine. Rather, this study does offer convincing evidence that, in so far as the chick is concerned, there is a definite continuity of the germ plasm from the gonads of one generation to the gonads of the following generation.

CONCLUSIONS

From the above discussion of the development of the germ plasm of the two sexes of the domestic fowl, the following conclusions may be summarized:

1. The primordial germ cells arise anterior and anterolateral to the head fold during the primitive-streak stage and migrate to the region of the future gonads, using the blood stream as the path of migration.

2. The primordial germ cells are easily recognized by their peculiar properties: large size, large clear-staining nucleus, and the presence of an attraction sphere and many yolk granules.

3. There are two separate proliferations of cords in the ovary. The cords of the first proliferation which form the medullary portion of the ovary carry a few primordial germ cells which take no part in this proliferation. The cords of the second proliferation, which occurs from eight to eleven days' incubation, are made up almost entirely of dividing germ cells and form the cortical region of the ovary.

4. There is only one proliferation of cords in the male gonad. This occurs from the sixth to the eighth day of incubation, as in the female, and they go to form the seminiferous tubules of the adult. There is a second period of activity in the male from fifteen to eighteen days' incubation dealing almost wholly with the germ cells and can be compared with the formation of the cords of the second proliferation in the female.

5. The sex of any age can be determined by the study of the chromosome complex. The male contains two large V-shaped chromosomes, while the female has only one.

6. A fixation solution, containing 15 parts 1 per cent chromic acid, 4 parts 2 per cent potassium dichromate, and 1 part concentrated glacial acetic acid, permits a definite identification of the chromosomes of the somatic tissue and young germ cells.

7. The synaptic stages of oogenesis occur from fifteen days' incubation to two days after hatching and the synaptic threads reappear about seventy days after hatching in the diploid number. Synapsis occurs in the male at the time the bird reaches sexual maturity.

8. The accumulation of yolk material in the ova sets in soon after hatching and continues until ovulation.

9. There is no evidence of a widespread degeneration of the primordial germ cells of either sex, and these primordial germ cells continue through to form the definitive germ plasm.

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EXPLANATION OF PLATES

The following figures were drawn with the aid of the camera lucida from sections cut 4, 5, and 7 μ and fixed with the solution previously described. All material was stained with Heidenhain's iron haematoxylin. Any combinations of objectives and oculars were used that seemed best, and the magnification was carefully calculated.

ABBREVIATIONS

AT.S., attraction sphere
BL.C., blood cells
C.C., cortical cords
D.O., definitive ovum
ECT., ectoderm
ENT., entoderm
FOL., follicle
G.E., germinal epithelium
INT.C., interstitial cell
L., lumen

M.C., medullary cords
MES., mesoderm
P.G.C., primordial germ cell
SEM.C., seminiferous cords
SEM.TUB., seminiferous tubule
STR., stroma
VIT., vitellus
W.B.C., wall of blood vessel
X., sex chromosome
YOLK, yolk material

PLATE 1

1 Section through the region anterior to the head fold of a fourteen-hour embryo showing the possible primordial germ cells still embedded in the entoderm. This indicates the great difference in size between the primordial germ cells and the somatic cells. Yolk material is common in all cells of this age. The metaphase plate showing the two V-shaped chromosomes indicates a possible male. This gives an oblique view of the sex chromosomes.

2 Section through the region anterolateral to the head fold of a sixteen-hour embryo, showing a primordial germ cell in the space where the mesoderm will later arise.

3 A primordial germ cell in a blood vessel of the splanchnopleure of a twenty-four-hour embryo, showing the blood vessel to be the path of migration.

4 Primordial germ cells in a blood vessel near the otic cup in a forty-six-hour embryo. This figure illustrates the relative size of the primordial germ cells and the blood cells. There are no germ cells found in the embryo proper until after the thirty-third hour of incubation; that is to say, until after the extra-embryonic and embryonic circulation anastomoses.

5 A primordial germ cell which has left the circulation at the wrong place. This was found in the head region of a fifty-five-hour embryo. Such misplaced cells are sometimes found in various parts of the embryo. Because of the presence of an attraction sphere, this cell is considered a germ cell rather than a local giant cell. Note the pseudopoda exhibited by this cell.

6 A metaphase plate from the neural tube of the same embryo as figure 3, showing one V-shaped chromosome indicating a probable female.

7 A section through the gonad region of a four-day embryo. The primordial germ cells are gathering in the stroma. The one-layered epithelium indicates a male.

8 The chromosome complex of the embryo figured in figure 7, showing the two V-shaped sex chromosomes which show this to be a male, as is indicated by the thinness of the germinal epithelium.

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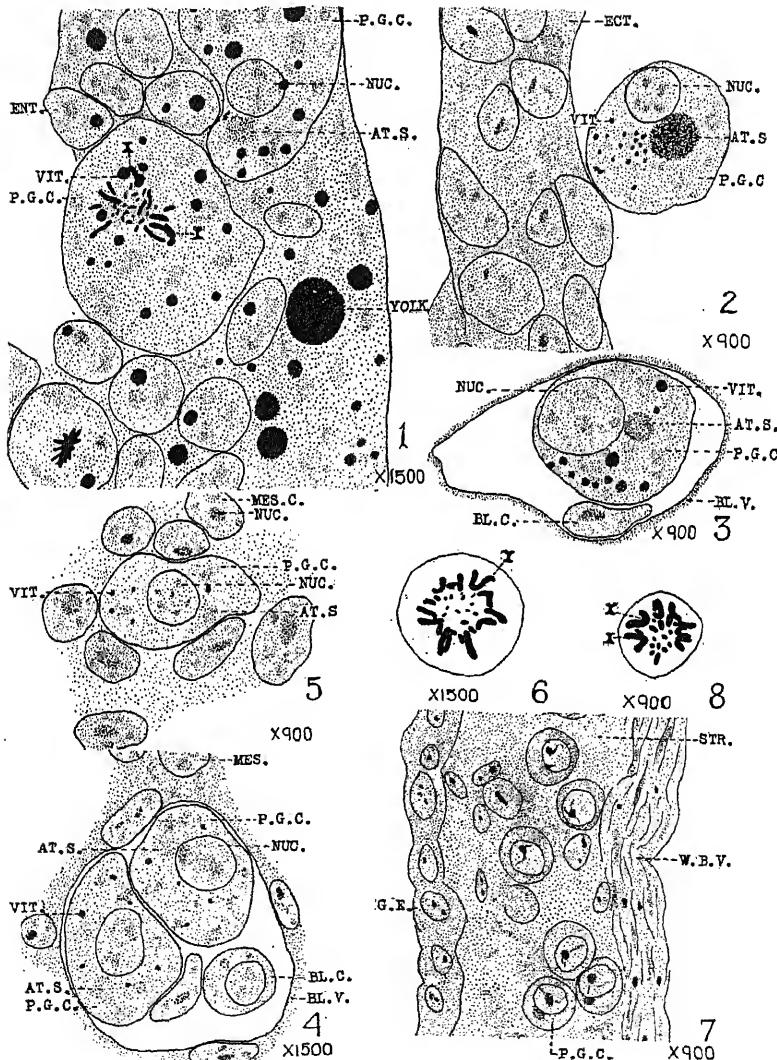
PLATE 2

9   A portion of the ovary of a seven-day embryo, showing the primordial germ cells placed in the germinal epithelium.   The thickness of the germinal epithelium is indicative of a female.

10   A portion of the ovary of a nine-day chick embryo, showing the full thirty-six chromosomes with the sex chromosomes indicative of a female.   Division of the germ cells is common in this age.

11   A portion of the ovary from an eleven-day embryo.   Division of the germ cells is still common at this age.

12   A metaphase plate from the same embryo as figure 11.   The one sex chromosome identifies it as being a female.

13   Section from the gonads of a fifteen-day female embryo.   This shows the first step in the maturation process which is setting in at this age.   The chromatin material is scattered in clumps throughout the cell and the nuclear wall has disappeared.

14   A low-power view of a section of the ovary of a sixteen-day embryo, showing the extent of the cortical cords and the appearance of the chromatin material during the normal synizesis stage which occurs in the fowl before the formation of the leptotene threads instead of after their formation.   Note that it is only the germ cells which are affected by this contraction.
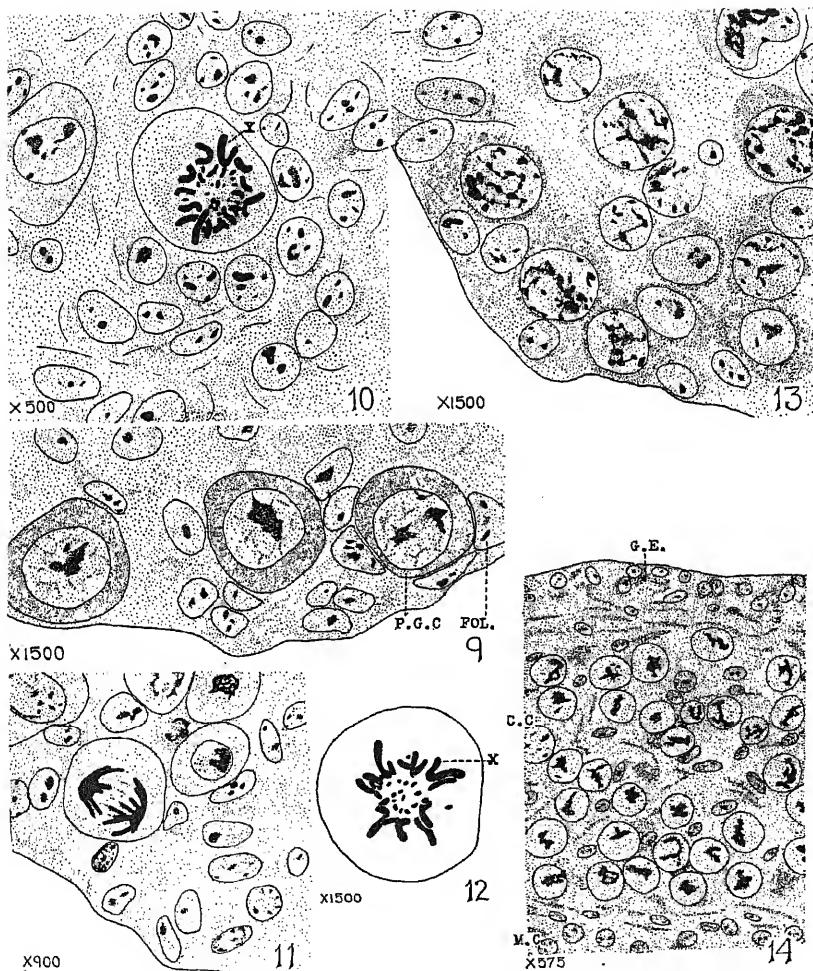
PLATE 3

15 A view of the synizesis with high power, to show the condition of the germ cells during this process. This figure was taken from the same embryo as figure 14.

16 A section from the gonads of an eighteen-day female embryo in which the synizesis stage is giving over to the spinning out of the leptotene threads. This appears very similar to the fifteen-day stage, except that the connective strands of chromatin material are more contrastive than in the fifteen-day stage. There are quite a few germ cells still in the synizesis stage, although the majority of them are unraveling at this age.

17 The germ cells of the twenty-day female embryo, showing the leptotene threads that have resulted from the unraveling of the synizesis of the sixteen-day stage. There is a slight hint of pairing of these leptotene threads that is to be investigated further. The threads radiate from the basal chromatin mass, showing the typical bouquet stage.

18 A highly magnified view of one germ cell from the female gonad two days after hatching. This shows the slow disappearance of the leptotene threads, the enlargement of the cell and the suggestion of the placement of the follicle cells into a ring. As can be seen, this cell is already considerably larger than the germ cells of the earlier stages, due to the accumulation of yolk material having already set in.

19 A section of the ovary from a ten-day chick, showing the enlargement of the definitive ova by the addition of yolk material. The follicle cells are aggregated into a definite ring around the ovum, while supporting cells may be seen outside the follicle ring.
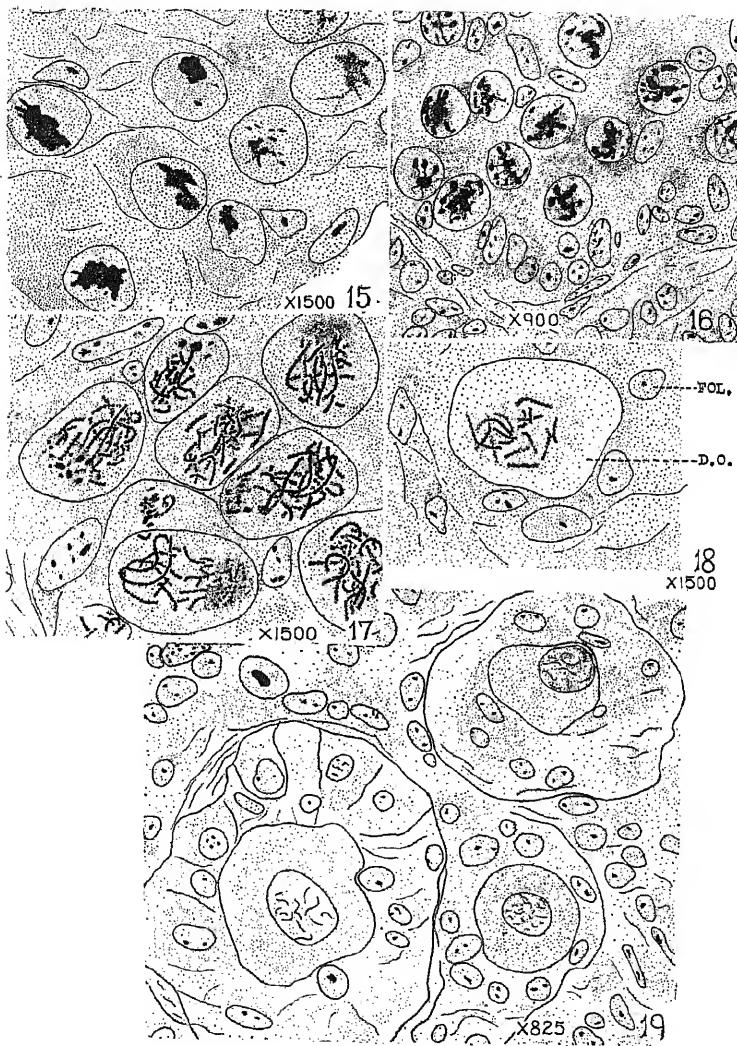
20 A photomicrograph of a single ovum from the ovary of a chick fifty-two days after hatching.

21 A section of the ovary from a sixty-five-day-old chick. Not as highly magnified as figure 20. Chromatin granules probably representing the chromatin threads are beginning to appear in this stage.

22 A highly magnified photomicrograph of the ovum from a sixty-nine-day-old chick, showing the reappearance of the chromatin threads of the pachytene stage. The threads are in the haploid number and are much heavier than the leptotene threads. The nuclear wall is broken down during this process.

23 A section through the ovary of a chick ninety-nine days old. Note the occurrence of much smaller ova in this same field.

24 A section through the ovary of a 115-day-old chick, showing the nucleus still in the center of the egg and further condensation of the follicle ring. The dark-staining yolk nucleus is shown placed to one side of the egg nucleus.

25 A section of an egg from a chick 180 days after hatching. Note the definite ring of large yolk granules near the periphery of the egg.

26 The adult ovary taken from an individual killed during the ovulation period, showing the condition of the sexually mature ovary. The various sizes of developing ova are to be seen. The supporting follicle stalks are shown attached to some of the larger eggs.
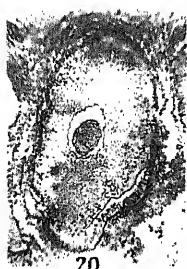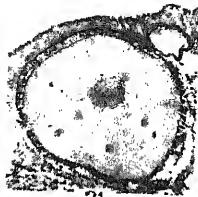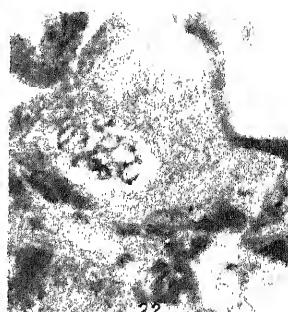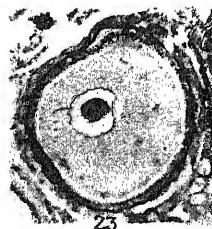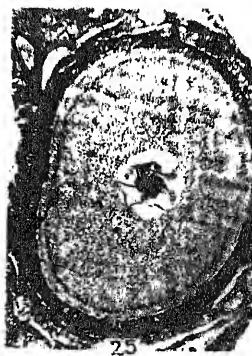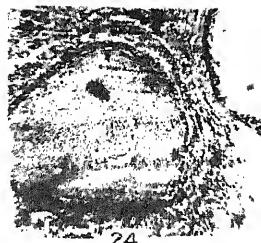
PLATE 5

EXPLANATION OF FIGURES

27 A section through the gonad of a five-day male embryo. The metaphase plate containing the two sex chromosomes show this specimen to be a male. Division of the primordial germ cell is very rare in this stage.

28 A section through the gonads of an eight-day male embryo, showing the ramifications of the seminiferous cords carrying with them the primordial germ cells. The germinal epithelium is thicker in this stage than in the previous stage.

29 A section from the testes of a twelve-day male embryo. This shows the first appearance of the interstitial cells in the stroma between the seminiferous cords. The cords take the stain less readily than in the earlier stages, resulting in the lighter appearance of these cords.

30 A section of a fifteen-day-old testis. Division among the primordial germ cells of this age is not uncommon.

31 A metaphase plate of a primordial germ cell in division from the same embryo as figure 30. The presence of the two sex chromosomes shows this sample to be a male.
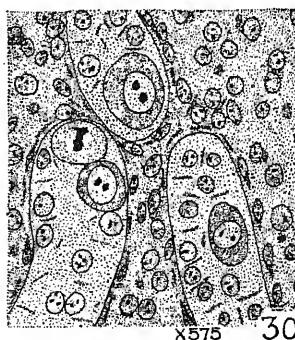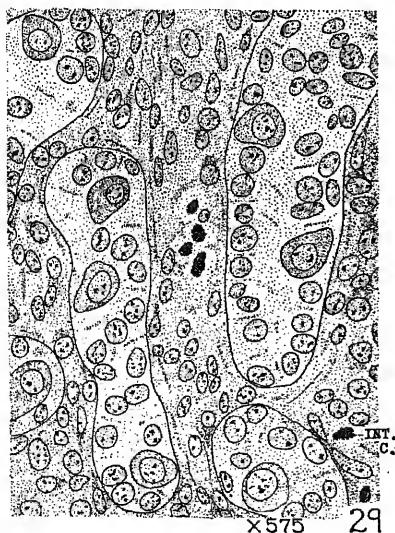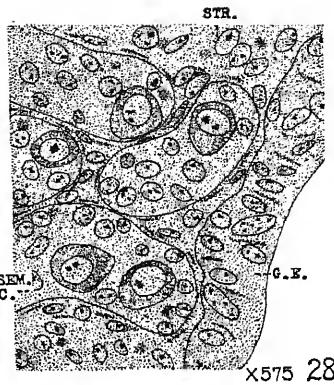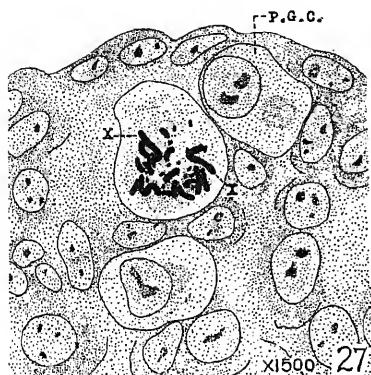
32 A section through the testes from an eighteen-day embryo, showing that the plane of cleavage of the primordial germ cells is not limited at right angles to the basement membrane of the cords, but can occur at any angle. The stroma is much thinner in this stage than in the previous ones.

33 A section through the testes of a chick two days after hatching. Note the very light appearance of the seminiferous tubules and the lumen which occurs for the first time at this age. Liquefaction of the peritoneal cells in the center of the cords is almost complete, so that all the cells, both peritoneal and germ cells, are arranged along the basement membrane.

34 A section through the testes of a chick twelve days after hatching, showing the enlargement of the lumen and the appearance of a syncytium.
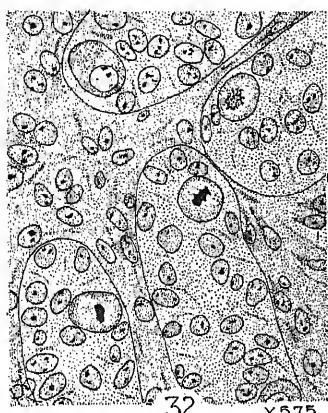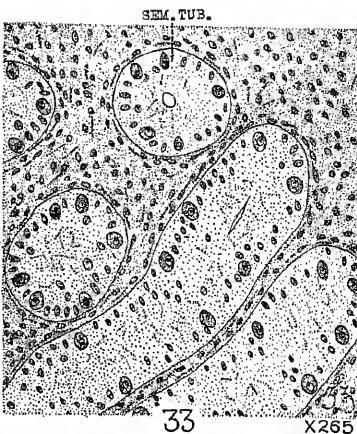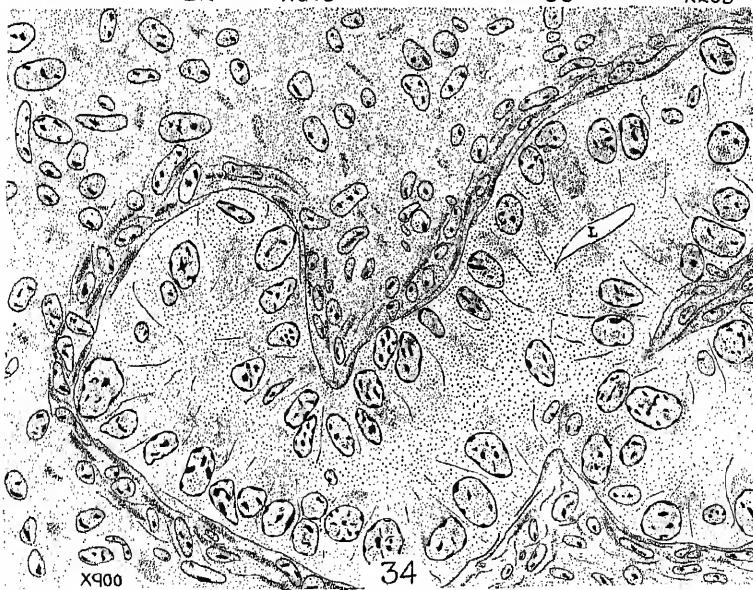
PLATE 7

35   A longitudinal section through a seminiferous tubule of a fifty-one-day-old chick.   The tubule is in the same condition as that of the preceding stage, except for the increase in size.

36   A cross-section of a seminiferous tubule of a ninety-six-day-old chick. The lumen is larger and the chromatin material of the cells of the tubule is aggregated into clumps of various sizes scattered throughout the nucleus.

37   A portion of a seminiferous tubule from a sexually mature bird, showing the resting gonial cells, the occurrence of synapsis, and the mature spermatozoon.
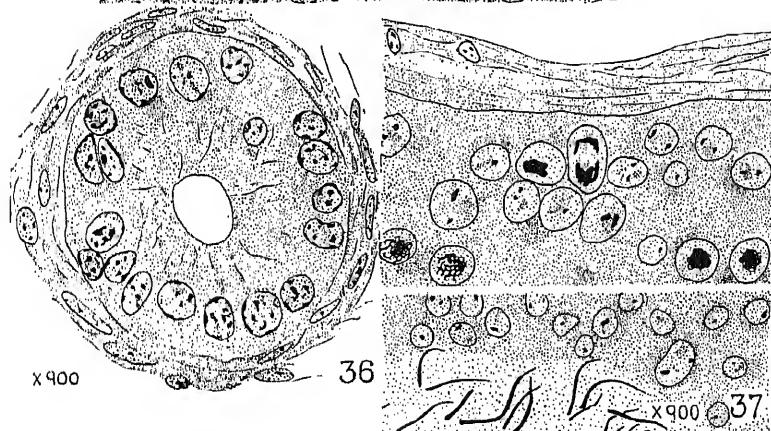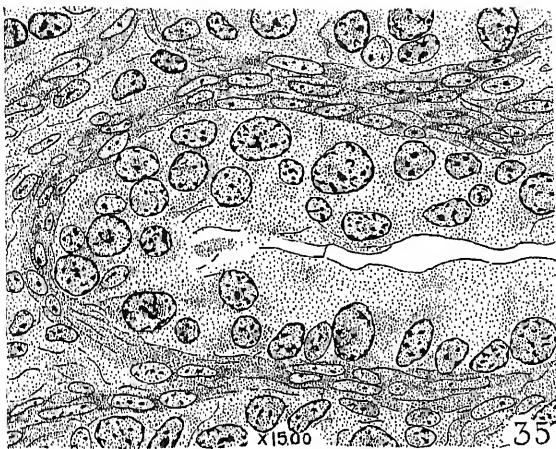
OBSERVATIONS ON THE GROSS AND MICROSCOPIC ANATOMY OF THE SLOTHS (BRADYPUS GRISEUS GRISEUS GRAY AND CHOLOEPUS HOFFMANNI PETERS)

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SEVEN HELIOTYPE PLATES (THIRTY-THREE FIGURES)

AUTHOR'S ABSTRACT

In this study observations are reported upon the morphological differences between the three-toed and two-toed forms of sloths, as well as a comparison made of some aspects between the sloths and the other groups of the Xenarthra. Much attention has been paid to the gross, as well as the histological examination of the viscera, musculature, and the vascular and lymphatic systems.

The importance of the correlation of the morphological findings with physiological studies has been emphasized. For example, the probable correlation of the vascular plexuses of the extremities with the postures and muscular activity of the different members of the Xenarthra is discussed. Much new information has been gained concerning the placentation and development of the sloths, as well as concerning the structure of the male and female reproductive tracts.

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INTRODUCTION

The morphology of the Xenarthra has been studied by numerous comparative anatomists, so that a rather extensive literature exists on some aspects of the anatomy of the sloth. Thus the skeleton, teeth, vascular system, and musculature have been repeatedly investigated. Other structures, such as the central nervous system, ductless glands, the viscera, and the reproductive tract, as well as the placentation and development, have been only inadequately studied. Moreover, all the existing work has been of gross morphological character, scarcely any microscopic observations having been undertaken. Furthermore, aside from the external characteristics and skeleton, which have been used for classificatory purposes, no careful comparison has been made between the anatomy of the three-toed and two-toed forms of sloths.

Since several laboratories have been established in recent years in tropical America, an opportunity has arisen to study more extensively the physiology and morphology of these extremely specialized and widely aberrant mammals. Something has been written about the sloth's habits by naturalists in the field and from occasional specimens kept in captivity in zoological gardens. Thus, observations upon its arboreal life, its posture, sluggishness, insensibility to fatigue, low body temperature, etc., have been recorded. But this inter-

esting mammal deserves more detailed study than was hitherto possible. Its physical organization differing in many ways from any other existing mammals, its well-nigh unique muscular activity and postural reactions, its metabolism and heat regulation present problems which can now be attacked.

Such investigations will necessitate a correlated knowledge of the gross and microscopic structure of the sloth with the physiological observations. With the end in view of studying the sloth at first hand in the living state and by securing tissues fresh and in ample amount by autopsy of recently killed animals, Dr. C. P. Richter and I spent several summers at the Barro Colorado Island Laboratory in the Panama Canal Zone. The writer conducted the morphological side of these investigations and obtained much new information upon the anatomy of the sloth, which it is the object of this paper to present. This survey of the morphology of the sloth is in no sense complete, the aspects chosen for study being those which bore a relationship to the immediate physiological problems. Thus much attention was paid to the histological examination of the tissues, more particularly to the muscle and ductless glands. A survey was also made of the morphological differences between the three-toed and two-toed forms of sloths, as well as a comparison of some aspects between the sloths and the other groups of the Xenarthra.

Some structures, for example, the skeleton, teeth, and gastro-intestinal tract, were largely neglected. These omissions are treated by reference to literature when such exists. It is hoped that the bibliography may serve as a key to the literature on the sloth, but it is borne in mind that much may have been overlooked, for the morphological accounts are often buried in comparative anatomical papers on mammals which do not reveal in their titles whether members of the Xenarthra were dissected or not. Moreover, the literature on the taxonomy and paleontology and early historical references to sloths are not fully presented, but references to the important recent articles on these subjects are given in which the complete literature may be found.

MATERIAL

The material for these observations was studied at the Barro Colorado Island Laboratory in the Panama Canal Zone. The sloths were purchased alive from the natives of the Canal Zone, who obtained them from the Zone or adjacent parts of the Republic of Panama. Two varieties of sloths were procured. They were identified from Goldman ('20) as the three-toed sloth, Bradypus griseus griseus Gray, and the two-toed sloth, Choloepus hoffmanni Peters. In all, some fifty animals were available for morphological work, of which three-fourths were of the tridactyl form, the remainder two-toed sloths. A large proportion of the tridactyl sloths were pregnant, so that a valuable series of fetuses was obtained. The animals were kept in an enclosure at the laboratory until experimented upon or killed for autopsy.

In addition to this material, the writer has had occasion to autopsy four specimens of the armadillo (Dasypus novem-cinctus) and to dissect two embalmed specimens of anteaters (Tamandua tetradactyla and Cyclopes didactylus), which were obtained through the courtesy of Mr. Gerrit S. Miller, Jr., of the U. S. National Museum. The study of this material has allowed certain comparisons to be made between the groups of the Xenarthra.

EXTERNAL CHARACTERS

It appears important to the author to preface this whole paper by a description of the difference in posture between the three-toed and two-toed sloths, because of the prevailing ignorance on this subject and the erroneous conclusions it has led to by comparative anatomists basing teleological arguments as to structure upon the mistaken belief that sloths universally spend most of their time hanging by all four limbs. These observations were possible because of the opportunity to study constantly the movements and postures of a number of living sloths in what amounted to a normal environment. Of the two forms considered, it is only true of Choloepus that it spends much of its time suspended equally

by all four limbs. Its front and hind extremities are nearly equal in length and it hangs habitually by them with its trunk in a nearly horizontal position. Bradypus griseus, on the other hand, scarcely ever hangs in the manner described for Choloepus, but spends most of its time asleep and waking in a squatting or sitting position, its hind limbs embracing a stem or a crotch of a tree, while its arms are free to be used for grasping foliage to be guided toward the mouth or for climbing. Hence, in the normal posture the trunk of Bradypus is perpendicular. When asleep, the hind extremities are used for support, the arms recline relaxed over the abdomen, and the head droops upon the chest or abdomen with the head and neck markedly bowed. Anatomically, also, the limbs are quite dissimilar from those of Choloepus. The fore limbs are extremely long, whereas the hind limbs are short. The proportion in the adult is as 10 to 7. This discrepancy in the relative length of the limbs is established early in uterine life, a fetus of 27 mm. (length of spine without tail over back) possessing fore limbs measuring 8 mm.; hind limbs, 5 mm.; another fetus of 36 mm. possessing limbs measuring 15 mm. and 10 mm., respectively. In Choloepus, on the other hand, the proportion of anterior extremities to posterior extremities is approximately as 10 to 9.5 in the adult and as 10 to 9 in a fetal specimen. In absolute length the fore limb of Bradypus is the longer, its hind limb the shorter of any of the extremities of the two genera. The great length of the fore limb of Bradypus is in great measure due to the relatively greater length of the humerus than in Choloepus. Furthermore, other anatomical differences are related most likely to the differences in posture. Thus the large calcaneus in Bradypus appears to be ideally qualified for aiding the foot in grasping upright limbs and in supporting the entire weight of the body in a perpendicular posture. In Choloepus, in which the body is usually suspended by the extremities, the calcaneus is correspondingly smaller. Moreover, in Choloepus, which assumes the hanging position almost constantly, the feet are provided with thick, rubbery,

hairless palms and soles, whereas in Bradypus the flexor surfaces of the hands and feet are covered by hair. Thus, there is a characteristic difference between the forms in reference to the length of limbs and the posture assumed by each (figs. 1, 2, 3, 4, 5, and 11).

It is interesting in this connection that Richter's studies ('26) have shown that their postural responses to decerebration are different. In none of the older papers (Brehm, '12; Menegaux, '09; Anthony, '07) is there a distinction made between the postures and climbing habits of the didactyl and tridactyl sloths. The present discrimination, however, should be extremely important in all further discussions of the correlation of morphology and posture in these animals. In the past it has been assumed in such discussions that the postures in the two forms are nearly identical. In this paper much of the reasoning on this old assumption will be shown to be fallacious. The foundation for the marked differences in the limbs and postures of the present-day sloths must be sought by future study of the fossil forms.

The external characters of the sloths of Central America in reference to their classification are described in an older work by Alston ('79), by Elliot ('04), and others, and, more recently, for Panama by Goldman ('20). A description of the external characters of Bradypus cuculliger cuculliger Wagler, of British Guiana, is given by Beebe ('26), whereas a description of the outward appearance of sloths in general without taxonomic value is given by Brehm ('12).

Of the two species under consideration in the present account, Bradypus griseus griseus, the three-toed sloth, possesses a conspicuous secondary sex marking in the male, the so-called 'saddle-mark,' an area of short, brilliantly colored hair over the dorsum. This is the only way that the males can be told externally from the females. The other species, Choloepus hoffmanni, the two-toed sloth, has no such distinguishing mark, and the author has found no means of discriminating the sexes externally.

The pelage of the adult tridactyl sloth consists of short, dense, silky under fur and of long coarse hair. In the region of the 'saddle-mark' the hair is absent, so that the mark forms a depression covered by short, matted under fur. The color of the mark is white or orange, with a median stripe of black fur dividing it into bilateral fields. The latter fields are often subdivided by an incomplete black bar, in some instances giving the appearance of a cross. In the female the dorsum is uniformly covered by coarse hair, but if the hair is clipped away the under fur reveals a concealed pattern similar to the 'saddle-mark' of the male. In a series of fetuses of the tridactyl sloth which was obtained it has been observed that the 'saddle-mark' is anticipated in both male and female fetuses, before the hair has erupted, by pigmentary markings in the form of a dorsal sagittal stripe of black pigment with white fields to either side broken up to some extent by small blotches of pigment. As the hair develops in fetuses of 130 to 135 mm. (length of spine without tail over back), the same relationship of markings is retained in both sexes. After birth, however, as the coarse outer hair develops, these markings become almost completely obscured in both male and female. The male cannot be distinguished from the female until, as puberty approaches, the outer fur disappears in the localized area over the dorsum, producing the characteristic male 'saddle-mark.'

The present material has given an opportunity to investigate the development of the hair in a series of fetuses of Bradypus, as well as to point out a possible relationship of the lines of hair direction to the postures in Choloepus and Bradypus.

In Bradypus griseus the hair begins to erupt in fetuses of 130 to 135 mm., beginning from a center of hair growth in the midline between the shoulders. In its further eruption the hair extends from this center upward, spreading over neck, head, shoulders, and down the dorsum of the arms. It also extends down the back from the primary center. Eventually, the hair extends from the shoulders onto the chest and

abdomen, directed caudally, and reaches the posterior extremities. Finally, the hair from the upper part of the back and hair sweeping around from the abdomen meet over the sacrum and tail in a whorl and raphé in which the hair is directed centripetally instead of centrifugally as in the primary center between the shoulders. A more detailed account of the development of pigmentation and the growth of the hair during fetal life will be found in a previous paper by the author (Wislocki, '27).

The direction of the pattern of the definitive hair coat of Bradypus griseus is identical with that established during fetal life, except that the hair of the neck and head in the adult tends to part in the dorsal midline anterior to the primary hair center. Considering the direction of the hair lines as described, in relation to the posture of the three-toed sloth, it is obvious that the pattern facilitates the maximum shedding of rain water to which the animal is frequently exposed. In keeping with such a conception is the fact that in the adult two-toed sloth which hangs by all four legs with the body horizontal, the hair direction is entirely different, the hair on the limbs lying in the direction from the ends of the extremities toward the dependent trunk and, on the trunk, from chest and abdomen toward the dorsum, where it forms a conspicuous fringe of long coarse hair lowermost of all. Unfortunately, there are no fetuses of two-toed sloths available to trace and establish the differences in the hair pattern more thoroughly. In the only fetal specimen of Choloepus (136 mm. length of spine without tail) in the present material, the hair has not erupted yet on the surface of the body (figs. 3, 4, and 5).

SKIN AND SUBCUTANEOUS TISSUE

The skin of the sloth is excessively thick and tough. In Bradypus the following measurements have been made: throat, 3 mm.; back of neck, 3 mm.; back, 2.5 mm.; dorsum of head, 2 mm.; legs, 2 to 2.5 mm.; abdomen, 2 mm.; skin over sacrum, $1\frac{1}{2}$ mm. In Choloepus, as follows: skin of

throat, 4 mm.; back of neck, 4 mm.; back, 3 mm.; dorsum of head, 3 mm.; legs, 1 to 2 mm.; abdomen, $1\frac{1}{2}$ to 2 mm.; skin over sacrum, 1 mm. The skin of limbs and abdomen is decidedly thinner in Choloepus than in Bradypus, and the epidermis, in general, is much less tough to the knife.

The skin of both forms is closely applied to the underlying musculature, so that it has little play and cannot be lifted in folds between the fingers. This condition is apparently due to the almost complete lack of loose areolar or adipose tissue between the skin and musculature. Hence, the dense fibrous corium is in close apposition to the aponeuroses of the underlying muscles and can be detached only by cutting, and not by manual separation, as is readily accomplished in some regions in other mammals. Although the skin is almost devoid of the possibility of being moved passively, the skin of the trunk must be subject to some active movement by the action of the extensive panniculus carnosus.

The panniculus carnosus of Bradypus has been studied by Zeiger ('25), who has found it to consist of broad bands of musculature, on the sides of the abdomen, arising from the ribs at the lower border of the m. pectoralis quartus and ending in the inguinal region and on the thigh. From a consideration of its location and shape, he ascribed to it an important rôle in reinforcing the abdominal wall and supporting the abdominal viscera in the hanging and climbing positions. It would be of interest to study the panniculus of the two-toed sloth with the object of comparing the panniculus in the two forms and attempting to correlate the findings with the differences in posture in the two animals which have been described in the present observations.

In histological sections the dense fibrous, inelastic character of the corium is apparent (figs. 22 and 24). Moreover, the connective tissue is relatively acellular, excepting along the blood vessels, which are surrounded by circumscribed sheaths of connective-tissue cells, which in section are seen as dense cellular bands traversing the corium (fig. 24).

Sweat glands are present in the two-toed sloth over the hairy portion of the body. In Bradypus the sweat glands, although present over the body surface, are extremely small and infrequent (fig. 22). In Choloepus sweat glands are extremely large and abundant over the hairless snout (fig. 24), but entirely absent in the naked sole pads of the fore and hind feet. These pads are lacking in Bradypus.

VOLUNTARY MUSCULATURE

The striated muscle of the sloth has never been studied microscopically, but deserves attention because of the remarkable physiology of the muscle. In the gross, in a freshly flayed cadaver, the muscle is found to be uniformly of the red variety, careful dissection revealing no difference in the color of any of the striated muscles. They are of a deep terra-cotta color regardless of whether the vessels have previously been washed out with salt solution or not. This coloration is in marked contrast to the smooth muscle which presents a grayish tinge. The diaphragm and the oesophagus down to the cardiac orifice of the stomach partake of the dark red color of the striated musculature. The impression has been gained that the musculature of Bradypus is slightly darker in hue than that of Choloepus. In several fetuses near term the muscles were found to have not yet acquired the dark red shade characteristic of the adult. It was impossible to ascertain at what age the muscle becomes dark, because of the lack of sufficient postnatal stages.

In the freshly killed specimen fibrillation of the muscle has never been observed. The idiomuscular contractions, however, are of interest. When one end of the muscle or a single fasciculus is stimulated mechanically by drawing a blunt instrument over it or pinching it, an extremely slow, vigorous contraction of the entire muscle or fasciculus is produced. Tetanic contraction is difficult to prevent on removal of the fresh musculature from the body.

Grossly, the muscles, particularly those of the extremities, are arranged in rather coarse fasciculi, which are readily

separable because of the rather scant and delicate epimysium and perimysium.

Microscopically, in fresh spreads, the muscles show cross-striations, exhibiting light and dark bands and Henle's and Krause's lines. The muscle has been studied in Bouin's fixed material with Mallory's connective-tissue stain, as well as by the Bielschowsky method. Both methods bring out the striations of the muscles excellently (figs. 18 and 21).

The muscle fibers appear to be all of the same size and character, except for the intrafusal fibers of the muscle spindle. A distinction between large and small fibers in the muscle cannot be made.

The nuclei of the adult muscle fibers show nothing unusual, unless it is a slightly more granular cytoplasm surrounding the nucleus than is ordinarily seen in mammalian muscle. The nuclei are extremely flattened and are almost invariably peripherally situated beneath the epilemma. In the muscle of late fetal stages, on the other hand, the nuclei are oval, frequently centrally situated, and invariably surrounded by a zone of finely granular cytoplasm.

The most striking feature of the adult muscle fiber is the large size and coarseness of the sarcostyles and their tendency to separate completely into individual fibers in the fixed preparations. Thus, the longitudinal striation of the muscle fiber is often quite as conspicuous as the cross-striation. Moreover, the presence of the coarse sarcostyles produces a characteristic beading of the cross-striations, due to a diminution in width of the cross-striations between neighboring sarcostyles.

Stained nerve endings in the muscle tissue by the Bielschowski method have revealed nothing unusual. Muscle spindles have been frequently observed in both haematoxylin and eosin and Mallory preparations. The individual spindle in sloth muscle is relatively very large, possessing a thick capsule and a relatively large lamellated intracapsular space (figs. 16, 17, 19, and 20). The intrafusal fibers are striated. Double spindles are not infrequently observed. Still larger,

lamellated structures, resembling pacinian corpuscles, occur frequently between the muscles and the periosteum in the neighborhood of the joints.

The uniformly red character of the striated musculature in the sloth appears to be its most characteristic feature. So far as the author is aware, this condition is not known for other mammals. Dissections of another member of the Xenarthra, freshly killed armadillos, revealed the usual appearance of both red and white striated muscles and muscles exhibiting both types of coloration.

VASCULAR SYSTEM

The vascular system of the sloth is of extraordinary interest, as the number of anatomical papers dealing with it bear witness (Carlisle, 1800, 1804; von Baer, '35; Hyrtl, '53; Zuckerkandl, '95; Hochstetter, '93, '98; Müller, '05, and de Burlet, '22). Its essential features consist of curious arterial and venous plexuses in the extremities and of a remarkable anatomical disposition of the vena cava which necessitates a circuitous return of the blood to the heart from the abdominal cavity and the hind limbs.

An examination of the arterial supply to the fore limbs of Bradypus and Choloepus reveals that the artery passes the first rib to enter the axillary space, giving off a branch to the neck. In the middle of the axillary space it gives off further branches to the walls of the axillary space. However, from the roots of these branches within the axillary space, in a distance not over 1 cm., a multitude of slender arteries are given off which surround and accompany the main axillary and brachial artery in its course down the arm. Thus a plexus is formed accompanying the artery as a series of arterial vessels which rarely anastomose. A lesser plexus is given off from the main plexus to accompany the radial nerve.

At the elbow the central artery continues into the forearm as a stout median artery which is accompanied by only a few accessory arteries. The large majority of the plexiform

arteries take the place of the interosseous artery between the radius and ulna. Toward the distal end of the forearm the plexiform arteries disappear or unite gradually until in the foot the dorsal arterial arch is formed as only a single artery. Similarly, the median artery, which possesses only a few secondary arteries, loses these completely in the forearm and reaches the foot to become a ventral arterial arch independent of any accompanying vessels. A detailed account of the course of the artery to the anterior extremity and the course and arrangement of its branches may be found in the papers by Zuckerkandl ('95 a, Choloepus) and Müller ('05, Bradypus).

A similar vascular picture is encountered in the hind limb. The aorta breaks into a median sacral artery and the common iliac arteries. The iliac in turn branches to form a hypogastric and an external iliac artery. Both of these latter become surrounded by arterial plexuses which accompany them in their further courses to the musculature of pelvis and extremity. The sacral artery also acquires an arterial plexus. A description of the minuter distribution of these plexuses may be found in the papers by Hyrtl ('53, Bradypus torquatus) and Zuckerkandl ('95 b, Choloepus).

These plexuses to the extremities are accompanied by corresponding venous plexuses which return the blood from the limbs, as was first discovered by Mayer ('39).

In the present study these plexuses have been investigated microscopically for the first time. In the arm, a cross-section in the region of the upper third of the brachial artery reveals the brachial artery surrounded by a plexus of arteries and veins (fig. 10). The artery is near the periphery of the plexus and exhibits the vascular tunics characteristic of so-called medium-sized arteries, possessing a well-defined media composed of smooth muscle separated from the adventitial coat by a sharply outlined elastica externa and from the intima by a narrow elastica interna. Associated with the artery is a bundle of smaller arteries and a set of veins. A count made in one instance of the number of vessels in the

vascular bundle reveals, besides the main artery, forty-three smaller arteries and twenty-five veins. These vessels are uniformly interspersed, together with the main artery, in a connective-tissue sheath that unites and incloses the vessels of the bundle. This stroma is composed of dense white connective tissue, with a sharp margin on the circumference of the bundle, contiguous with the surrounding looser connective tissue of the arm.

The smaller arteries are of nearly uniform size and shape, some six to eight of them being the equivalent in circumference and capacity of the brachial artery itself. The small arteries, too, possess the character of medium-sized arteries, exhibiting a muscular media bounded by an elastica interna and externa. The sections reveal, as was already known from dissection, that the arteries scarcely ever anastomose, simply running parallel to the primary artery for a variable distance until they become deflected to supply muscles of the arm.

The veins, on the other hand, which are regularly interspersed among the arteries, are fewer in number, four to six times the capacity of the arteries, and anastomose frequently, producing a true venous plexus. There is no main venous trunk corresponding to and accompanying the brachial artery, the veins being all of about the same caliber.

In addition to the arteries and veins, the vascular bundle conveys large lymphatic trunks about equal in number to the veins. These also are interspersed fairly uniformly among the arterial and venous trunks.

The microscopic relationships recorded here hold true for the plexuses of the lower extremity and pelvis as well. There appears to be nothing unusual in the ultimate peripheral capillary distribution of the vessels to the musculature of the extremities.

The significance of these plexuses in the extremities of the sloth is a moot question. Carlisle (1800, 1804), who first described them, expressed the belief that they were causally related in some way to the slow movements executed by the sloth. The question became complicated, however, by

the fact that it was soon discovered by comparative anato-
mists (von Baer, '35; Mayer, '39; Hyrtl, '54; Zuckerkandl,
'95; and Müller, '05) that the related forms, Dasypus and
Myrmecophaga, as well as mammals in no wise closely related
to the edentates, possess vascular plexuses in the extremities.
Thus, similar, although by no means identical, vascular ar-
rangements have been described in certain marsupials, ceta-
ceans, pinniped carnivores, rodents, and lemurs.   Vrolik
(1826) sought to establish the thesis that arterial bundles are
characteristic of climbing animals, serving the purpose of
preventing any interference with the circulation by pressure
from the greatly contracted muscles.   Von Baer ('35) dis-
misses the ideas of Carlisle and Vrolik as improbable and
points out that the arterial networks such as he described in
Phocaena communis resemble the embryonic pattern which
appears to have persisted to a large extent in the adult
organism.

In discussing the possible function of plexiform arterial
structures, Hyrtl ('54) distinguishes two anatomical pat-
terns, a diffuse radiate pattern, characteristic of monotremes,
which he examined, and a massive network type which sur-
rounds a main artery as in the Bradypodidae.   The former,
he finds, occurs in those animals which do heavy muscular
work, but of the nature of oft-repeated quick movements,
such as burrowing animals, for example, the armadillo.
Whereas the latter type of vascular arrangement occurs in
mammals, such as the sloth, in which the movements are
slow and posture prolonged.   The advantage of this arrange-
ment in these latter animals, he believes, would be to prevent
the contracted muscles from interfering with the circulation.
Correlated with his discussion of the types of blood vessels
in reference to muscular activity, he points out that, in the
sloth and armadillo, only those arteries exhibit plexiform
patterns which are associated with and destined to be dis-
tributed to the musculature, whereas the arteries which sup-
ply the viscera or the bones, periosteum, skin, and subcutane-
ous tissue of the limbs are invariably single twigs.

Müller ('04), in exercising a critique on this subject, rejects all of the explanations offered, based on the doctrine of adaptation to purpose. He believes it is conceivable that more archaic forms of animals possessed more extensive arterial paths than present living ones. He points out the similarity between the embryonic vascular pattern and the pattern of the vascular plexuses found in the adult, but emphasizes the lack of complete homology. Moreover, although the network may represent the oldest phylogenetic form, this does not explain its persistence in certain animals (Didelphys, Halmaturus, Hydrochoerus, Phocaena, Edentata, Stenops tardigradus, and Pinnipedia) and not in others. Müller concludes that purely mechanical conditions during ontogeny are the primary cause of the formation of such networks.

In reference to their etiology, Müller decides that a distinction must be drawn between the simple network and the bundle pattern. The former represent a persistence of the embryonic condition. The bundle form, such as is encountered in the Bradypodidae, he regards as organs which have developed in a specialized direction. The causative factors in the specialization, however, remain obscure.

As to the function of arterial networks, Müller says that animals with well-developed arterial bundles in the extremities, such as Bradypus, are extremely slow in their movements. Further, he quotes Braune to the effect that muscular activity has been demonstrated to play a considerable rôle in aspirating the venous blood from the extremities. In Bradypus, which he claims passes much of its existence as though dead, the aforementioned influence on the veins has been lost, hence one must look for other means to explain the venous flow. This he believes to have found in the vascular bundle, in which he postulates an aspiration of the blood in the veins due to the pulse in the adjoining arteries. He finds a simile in the comparison to a sponge which is periodically pressed out. This whole explanation seems extremely weak.

The writer has not only investigated the arterial plexuses in the sloths, but has observed the vessels of the extremities in the entire group of Xenarthra. The fact that Tamandua and Cyclopes were drawn into the comparison, besides Dasypus and Myrmecophaga, the vascular patterns of which were previously known, has led in the present investigation to a grouping of the Xenarthra on the basis of the vascular pattern. Moreover, this grouping can be correlated with a parallel grouping of the Xenarthra, based on the observed postures, movements, and muscular activity of the animals. This correlation will afford, perhaps, eventually a key to the solution of the function of the vascular plexuses in mammals. Furthermore, the grouping, on the basis of a study of the blood vessels, appears to be in harmony with the relationships of the Xenarthra arrived at from the study of other anatomical characters.

As has been described, the sloths possess highly differentiated vascular bundles in the extremities which form conspicuous cords of plexiform arteries and veins principally in the upper arms and thighs. In the armadillos, on the other hand, the vascular plexuses are very much simpler in character, consisting at the most of loose, wide-meshed vascular anastomoses in the vessels, particularly of the forearm and leg. In Myrmecophaga the vascular anastomoses in the extremities are scarcely more developed than in the Dasypodidae. In Tamandua and Cyclopes, however, the vascular patterns become increasingly more complex, constituting a transition from the condition present in Dasypus and Myrmecophaga to the highly specialized structure characteristic of the Bradypodidae. In Tamandua the vascular anastomoses are increasingly more numerous than in Myrmecophaga, especially so in the upper arm and thigh, while a Cyclopes dissection reveals vascular bundles in the extremities, equally complex to those of the Bradypodidae.

Concerning the postures, movements, and muscular activities, the author has had occasion to observe all of these animals in the living state. It is obvious at once that in posture

and locomotion Cyclopes resembles the sloths very closely and is nearer to them than any other of the group. Tamandua, which is also arboreal, exhibits in its native state much of the deliberate slow quality of movements characteristic of sloths as well as the ability to climb along the undersides of limbs of trees. Myrmecophaga, on the other hand, is terrestrial and shows in walking and movements none of the characters of the Bradypodidae. The armadillos, finally, are also ground-living, very active in running and burrowing, and show no trace of any relationship to the sloths in postures or movements.

There appears, then, to be a correlation between the vascular structures in the extremities of the Xenarthra and their muscular activities and postures, the three forms, Bradypus, Choloepus, and Cyclopes, which exhibit a high degree of ability to maintain postural tone and to execute slow movements, being possessed of a highly specialized arrangement of the vessels to the extremities. The active terrestrial forms, Dasypus and Myrmecophaga, on the other hand, possess simple vascular networks of an entirely different character, while Tamandua occupies an intermediate position as regards activity and vascular arrangement.

It is of interest in this connection that the musculature of the Bradypodidae has been found in the present investigation to be exclusively of the red kind—a unique condition among mammals. In Dasypus, on the other hand, the muscle is found to be red and white, either in the same or separate muscles—the usual condition in mammals. It will be of interest when fresh material of the anteaters becomes available to see whether the members of the genus show intermediary positions in reference to the relative amounts of the red and white varieties of muscle.

What the particular function is of the vascular bundles, seen characteristically in Bradypus, Choloepus, and Cyclopes, we cannot say until physiological studies acquaint us more with the functional needs of the musculature in these animals. The vascular bundles possess the character, grossly and

microscopically, of tough, relatively incompressible tubes or pencils of dense connective tissue containing the blood vessels. In view of what has been said of the movements of these animals, it may well be possible that the arterial bundles insure a steady flow of arterial blood to the extremities which might otherwise be interfered with by the pressure exerted by the slow and protracted contractions of the muscle if only a single artery were present. It may be recalled that the vascular bundles occur only in the deeper portions of the extremities between the muscle masses, and not in the skin or distal portions of the legs, nor do the plexuses involve the individual muscles themselves in any unusual way.

It has been pointed out frequently in the literature that arterial plexuses reduce the rate of flow to the extremities. However, this would be of no consequence physiologically, if the volume reaching the limb were at the same time increased. Whether the volume of blood delivered to the limb through the arterial plexus is relatively greater than in an animal of comparable size possessing a single artery has never been determined.

The other interesting phase of the anatomy of the vascular system of sloths concerns the posterior vena cava. Hochstetter ('93, '98) found that it is the rule for edentates to possess a double vena cava. However, in Choloepus he observed, besides the duplicity of the vena cava, certain interesting peculiarities in its branches that have not been described in any other animal except the sloth. Each of the two posterior caval veins arises from a venous plexus which accompanies each iliac artery. It is surprising, however, that these plexuses are much greater in caliber than the trunks of the posterior venae cavae to which they give rise. This is explained by the fact that each of the iliac plexuses gives off two branches, nearly as large as the caval trunks, which enter the vertebral canal via the first two ventral sacral openings and deflect a good part of the blood into that channel (fig. 6).

The venae cavae course on either side of the aorta to the region where the renal arteries are given off from the latter; at this point the two veins unite to form a single trunk to the right of the aorta. In spite of the fact that in its course the vena cava has received renal, spermatic, and adrenal tributaries, it dwindles to almost nothing in its upper course before it reaches the liver. Only within the liver does it again become augmented in caliber.

It is clear from the foregoing findings that only a part of the blood is returned to the heart from the hind part of the body through the vena cava. The explanation of this is that the posterior caval veins connect with the intravertebral veins by rather large vessels which enter the intervertebral foramina. Thus, the anastomosing chain of intravertebral veins uniting the circellus venosi on the right side has been converted in Choloepus into a tremendous venous trunk. This trunk extends uninterrupted to the level of the tenth and ninth thoracic foramina through which it leaves to connect with the proximal portion of the vena azygos whereby the blood is conducted to the heart. The vena azygos, except for its proximal end, as well as the vena hemiazygos, could not be found.

Hochstetter has also examined Bradypus and has found essentially the same condition of the vena cava as in Choloepus, namely, a double posterior vena cava and the presence of branches entering the vertebral canal which give rise to an intravertebral trunk which passes cephalad to connect eventually with the anterior vena cava principally through the sixth and seventh thoracic intervertebral foramina on the right side. The vena azygos, except for its proximal end, and the vena hemiazygos are absent.

The two posterior venae cavae differ from those described in Choloepus in that they are connected caudally by plexiform anastomoses. Moreover, contrary to the finding in Choloepus, the vena cava, after the union of the two separate trunks at the level of the renal arteries, does not diminish perceptibly in caliber. Hence the anastomotic pathway via

the vertebral veins appears to be less pronounced in this species.

Hochstetter attempts to explain the curious condition of the vena cava and its anastomoses in sloths on the basis of the influence of posture and gravity on the return of the blood to the heart. He postulates that, in the hanging position, gravity favors the return through the vertebral pathway, whereas it diminishes the mechanical usefulness of the caval and azygos channels. He attempts, moreover, to explain the discrepancy in the observed size of the vena cava in Choloepus and Bradypus on the basis of differences in posture in the two forms, based on measurements of their extremities. He observes that the hind legs in Bradypus are markedly shorter compared to the front legs than in Cholocpus. Hence he thinks that, in the hanging position, the pelvis of Choloepus would be nearer the horizontal than in Bradypus; therefore, in Choloepus, in the absence of gravity as an aid to propelling the column of blood through the vena cava, the rate of flow would be diminished and the blood would have to seek other channels for reaching the heart. An added factor, he believes, is possibly the greater relative length of the abdominal segment of the vena cava in Choloepus than in Bradypus.

Hochstetter's interpretation, although ingenious, does not take cognizance of the actual postures as observed in living sloths and described in the first part of this paper. Moreover, a recent study by de Burlet ('22) on the veins of sloths makes Hochstetter's view even more improbable.

De Burlet had the opportunity to study the venous system in a fetus of Choloepus in which he was able not only to verify, but to extend Hochstetter's observations upon the relationship of the vena cava to the intravertebral veins. He found that, in addition to the lumbar and intercostal veins, the vena cava anastomoses with the intravertebral veins via two larger veins (venae basi vertebralis) which perforate the bodies of the lumbar and thoracic vertebrae. Furthermore, by sectioning the fetus, he demonstrated the tremendous

longitudinal intravertebral vein which conducts the blood toward the heart. It is fully of the same magnitude as the spinal cord itself and situated on the right side. As a result of this, the spinal cord and its membranes are displaced to the left side of the vertebral canal. The segmental spinal nerves pass into the intervertebral foramen dorsally to the vein. The blood leaves the spinal vein through the eighth to twelfth intervertebral foramina, uniting as the azygos vein to drain into the superior vena cava. In a fetus of Bradypus de Burlet found essentially the same venous arrangement in the vertebrae and spinal canal as in Choloepus.

The lumbar and thoracic vertebral bones of adult Bradypodidae show the osseous foramina through which the enlarged perforating veins passed. Other Xenarthra do not possess comparable openings in the vertebrae. However, examination of the vertebrae of fossil sloths (Scelidotherium, Mylodon, Grypotherium) reveals indubitable evidence of the existence of large perforating veins and of a copious intravertebral circulation which emerged through several enlarged thoracic intervertebral foramina.

These observations show that the venous pathway through the vertebral canal occurred in the ground-living ancestors of the present-day sloths. Hence, as de Burlet points out, the condition cannot be explained on a mechanical principle considering the postures of the present-day sloths alone.

LYMPHATIC SYSTEM

Concerning the lymphatic system of the sloths, nothing is given in the literature. The author had the opportunity to investigate the lymph channels by injection in fresh cadavers of Bradypus. In the fresh, abundant lymphatic plexuses can be filled by stab injections of India ink into many of the viscera. Thus, injections into the serous coat of the intestine fill large lymph channels in the mesentery, abundantly supplied with valves. These drain into lymph nodes which occur in great abundance the whole length of the mesentery. Characteristic of these nodes is that they are small, brownish, and

discrete, being on an average no more than 4 mm. in length. They are markedly different from the large mesenteric nodes seen in Carnivora and Ungulata. Lymphatics can also be injected with ease by stab injections into the testis or kidney. From the latter organ, lymph plexuses fill unusually well to constitute two sets, one an abundant retroperitoneal set, the other an anastomosing network which follows the ureter from the renal pelvis to the bladder. The latter observation is of interest in that in this animal, contrary to the belief concerning other mammals, the lymphatics accompanying the ureter are oriented from kidney to bladder. The other interesting aspect of these observations is that lymphatics can be injected so readily from the kidney—a feat difficult to accomplish in the ordinary laboratory mammals, because the injection invariably enters veins instead of filling lymphatics.

If the abdominal lymphatics of Bradypus are filled in one of the aforementioned ways or by injection into an inguinal or retroperitoneal node, and the course of the injection followed, it is discovered that the lymph trunks communicate freely through a number of openings directly with the abdominal veins, most frequently with the vena cava, but also with the portal vein. Moreover, careful dissections of these preparations reveal that Bradypus possesses no thoracic ducts in communication with the abdominal lymphatics and that all of the lymph from the lower extremities and from the abdominal viscera enters the veins of the abdomen. Some of the lymph enters the liver directly via communications of lymphatic trunks with the portal vein, and one of the features of an injection of the abdominal lymphatics in the sloth is the immediate appearance of ink in the periphery of the hepatic lobules in the neighborhood of the entrance of the portal vein. The condition is very similar to the one described by Job ('15) in the rat and by Silvester ('12) in certain South American monkeys in which the lymph from the hind portion of the body enters the venous system by portal or postcaval openings. However, in Bradypus there appear to be fewer openings into the renal veins than in the Simiae described

by Silvester. These lymphatics, draining permanently into
the abdominal veins, suggest a multiple origin of lymphatics
from the abdominal veins. It is known from the embryology
of the lymphatics (Sabin, '13, '16) that the abdominal lym-
phatics commence as a retroperitoneal sac in communication
with the abdominal veins. Although it is not definitely
known, it is thought that as the thoracic duct becomes es-
tablished these retroperitoneal communications of the lym-
phatics with the vena cava and its tributaries become lost in
most mammals. The persistence of permanent communica-
tions in some mammals, such as the sloth, indicates a per-
sistence probably of an embryonic condition.

The lymphatics of the extremities have been injected in
several sloth fetuses. In two such specimens (length of
fetuses, 10 and 11 cm.) two lymphatic plexuses have been
recognized; one, a superficial plexus injected from the sub-
cutaneous tissue of the foot which drains into trunks which
course beneath the skin to empty into lymph nodes in the
axilla; the other, a deep plexus accompanying the arterio-
venous bundles of the extremity and draining into deeper
nodes of the axilla and neck.

The lymph nodes of the adult sloth are variable in char-
acter. In general, they are small, though numerous. As
has been pointed out, the mesenteric nodes are never over
4 mm. in length, discrete, and abundant. The remaining ab-
dominal nodes and the thoracic nodes are also small. The
largest nodes have been encountered in the axilla and groin,
several measuring up to 1 cm. in length. No so-called hemal
nodes nor hemolymph glands have been encountered.

THE ABDOMINAL VISCERA

The peritoneal cavity

The peritoneal cavity of the freshly killed Bradypus con-
tains a few cubic centimeters of clear fluid. In both Bradypus
and Choloepus much of the cavity is taken up by the enormous
stomach. The bladder is usually markedly distended with
urine. The omentum is small and contains very little fat.

The paucity of adipose tissue in both Bradypus and Choloepus is striking. It has been pointed out already that there is scarcely any subcutaneous fat excepting in the axilla and groin. Fat is also scarce in the body cavities. The omentum contains very little and there is practically none in the mesentery, the mesenteric vessels and lymphatics being unaccompanied by sheaths of adipose tissue as is the rule in other mammals. The only marked deposit of adipose tissue occurs about the retroperitoneal organs, the aorta, kidneys, and urogenital passages. The body fat of Choloepus exhibits a curious orange or buff color, whereas that of Bradypus is a cream color.

Gastro-intestinal tract

The stomachs of Bradypus and Choloepus are complex. They have been studied by Klinkowström ('95) and Oppel ('96) to whom reference is made for information concerning them. Klinkowström has compared the two forms.

The only observations made on the stomach in this study concern its size and weight. In sloths in their normal habitat the stomach contains a tremendous quantity of food in the form of cecropia leaves which have been masticated to a pulp. The stomach contents weighed 662 grams in a three-toed sloth of 4316 grams total body weight—a ratio of 1 to 6.4; in another instance, 493 grams to 3121 grams total body weight—a ratio of 1 to 6.3. In a specimen of Choloepus the stomach contents weighed 570 grams out of a total body weight of 4134 grams—a ratio of 1 to 7.2. Furthermore, it has been noted that the red muscle of the oesophagus extends to the cardiac orifice of the stomach.

No observations have been made on the small intestine, except to note that in Bradypus griseus the duodenum invariably contains a quantity of nematode worms. These have never been found in Choloepus in the present series.[2]

[2] These parasites have been identified by Dr. G. Steiner as Leiuris decodontus n.sp. The species is related to two other forms, already described, from tridactyl and didactyl sloths.

In seeking for an explanation for this condition he postulates that the hanging posture of the sloth may have been conducive to producing it. However, this seems rather unlikely in view of the fact that the anatomical peculiarities of the liver are common to both Bradypus and Choloepus, whereas, as has been described under the external characters of the sloth, the postures of the two animals are so different that it can scarcely be assumed that they could give rise to identical morphological changes in both animals. It seems more likely that the condition of the liver is either inherited from fossil ancestors or is the result of other factors in Bradypus and Choloepus themselves. If inherited, it is improbable that the force of gravity in the hanging position played an etiological rôle, for, so far as can be postulated from the study of fossil forms, the posture of those animals was not a hanging one. It is presumed to have been one in which the lower extremities were used primarily for squatting or standing, whereas the upper extremities were used for grasping the branches and foliage of low trees to bring them within reach of the mouth. It is obvious that the hypothetical posture of the ground sloths resembled that of Bradypus more than that of Choloepus.

It may be suggested that a factor within Bradypus and Choloepus themselves bears investigation in this connection. The stomach adapted to a herbivorous diet is extremely large and, as de Burlet points out, influences the shape of certain lobes of the liver. It would not be unpromising to continue on the assumption that the stomach has not only modified the form, but has also produced the curious rotation of the liver in the Bradypodidae. This assumption seems also promising in view of the fact that the stomach is specialized in an identical manner in all of the Bradypodidae, whereas in the nearly related families of Myrmecophagidae and Dasypodidae, in which the specialization has not occurred, the modifications of the liver are also lacking.

The gall bladder

The absence of the gall bladder in Bradypus, its presence in Choloepus, was first mentioned by Cuvier, and has since then been amply affirmed.

The gall bladder has been examined in six specimens of Choloepus in the present series. The most striking thing about it in the gross is its small size and simple, straight neck (fig. 13). It is located on the intestinal facies of the liver in the central lobe. About two-thirds of it is buried in the parenchyma of the liver. In five of the six specimens the bladder is confined entirely to the intestinal face of the liver; in the sixth specimen, in which the bladder is larger than in the others, its free pole reaches the edge of the liver and is just visible on the diaphragmatic surface. The outside dimensions of the five smaller vesicles are approximately 15 mm. length and 9 mm. diameter. In the fresh approximately 1 cc. of dark green bile can be removed from the bladder. On cutting it open, it is found to be relatively thin-walled, the mucosa thrown everywhere into delicate elevations. In one instance there is a delicate, free thread of tissue which bridges the fundic pole from wall to wall. In another specimen there is a tiny tubular prolongation of the lumen of the fundic pole which extends toward the edge of the liver for 3 mm. to end in a fibrous thread which extends some 3 mm. farther. The simple, funnel-shaped orifice of the gall bladder is protected by two semilunar folds. A dissection of a further specimen reveals that the cystic duct is 2 cm. long. It joins the common duct just after the latter has formed by the union of three hepatic ducts.

Microscopically, the gall bladder exhibits rather feebly developed coats; the musculature especially is extremely scant in amount (fig. 23). As Halpert and the writer ('27) have pointed out, the muscle is unevenly developed, being more plentiful on the peritoneal surface than on the embedded surface.

Examination of the gall bladder in the other members of the Xenarthra reveals that Dasypus possesses an extremely

large, complex L-shaped gall bladder, whereas Tamandua and
Cyclopes exhibit gall bladders resembling in shape that of
Choloepus, being vesicular bladders tapering by a short,
straight, funnel-shaped neck into a straight cystic duct. The
gall bladder of Tamandua, although similar in pattern to
that of Choloepus, is markedly larger and better developed,
possessing a capacity about four times that of Choloepus.
The gall bladder of Cyclopes is similar to that of Choloepus,
but diminutive. However, in comparison to the relative sizes
of the two animals, it is undoubtedly better developed than
in Choloepus. From a consideration of the relative size and
complexity of the gall bladder in the respective groups, it
seems not unlikely that the gall bladder represents in Choloe-
pus a degenerative or transition form from mammals pos-
sessing a well-developed gall bladder, such as Dasypus,
through others, such as Tamandua and Cyclopes, in which it
is relatively simple, to an animal such as Bradypus, in which
it no longer appears.

The spleen

Bradypus	Absolute weight of spleen, grams	Grams spleen per kilo of total body weight	Grams spleen per kilo of corrected body weight
1	5.1 (adult)	1.09	1.32
2	4.5 (adult)	1.03	1.29
3	3.5 (adult)	0.81	1.00
4	3 (juvenile)	0.97	1.22
Choloepus			
1	7.4 (young adult)	1.8	2.1

No observations of any significance have been made upon
the spleens in this study. In the gross the spleens of Brady-
pus and Choloepus differ markedly in shape (figs. 8 and 14);
that of Bradypus is always irregularly tubular, consisting
usually of one mass, although semidetached or accessory
splenic nodules have frequently been observed in the omentum
and neighboring pancreas. The spleen of Choloepus, on the
other hand, is roughly triangular and markedly flattened.
At the hilum it is intimately fused with the adjoining pan-
creas. In Dasypus and Tamandua the spleen is similar in

configuration to that of Choloepus. In Cyclopes, on the other hand, it is slender and tubular.

The pancreas

Bradypus	Absolute weight of pancreas, grams	Grams pancreas per kilo body weight	Grams pancreas per kilo of corrected body weight	Ratio of pancreas to corrected body weight
1	6.3 (adult)	1.46	1.81	1 to 552
2	4.7 (adult)	1.09	1.33	1 to 750
3	5.45 (juvenile)	1.72	2.25	1 to 445
Choloepus				
1	5.3 (young adult)	1.28	1.53	1 to 651

No gross or microscopic studies of especial note have been made on the pancreas. Compared to the other abdominal viscera, the pancreas is large. In both Bradypus and Choloepus it is fleshy, compact, and yellowish orange in color. Islets of Langerhans are typically present in the microscopic sections.

The adrenals

Bradypus	Absolute weight of both adrenals, grams	Grams adrenal per kilo total body weight	Grams adrenal per kilo of corrected body weight
1	0.65 (adult)	0.139	0.169
2	0.55 (adult)	0.109	0.128
3	0.6 (adult)	0.140	0.170
4	0.4 (juvenile)	0.128	0.163
Choloepus			
1	0.7 (young adult)	0.170	0.205

The adrenals in the adult are small by comparison with other mammals of comparable size. In both Bradypus and Choloepus they are situated high up in the abdomen to either side of the aorta.. They are not associated with the kidneys, the latter organs occupying a pelvic position.

In fetuses of the sloth the adrenals are surprisingly large and composed mainly of cortical tissue. In a fetus of Choloepus (length, 136 mm., spine without tail) the adrenals are 18 mm. long, nearly cylindrical in shape, and 6 mm. in diameter. They have at this stage attained already their full adult size. They surpass in volume by four or five times

the fetal kidneys which are situated caudally to them. They extend from the lower border of the liver to the brim of the pelvis. In the fetus the lower poles of the adrenals almost touch the upper poles of the kidneys, but in the adult the adrenals come to lie high up in the abdomen near the coeliac axis, while the kidneys, retaining a pelvic position, become widely separated from them.

On gross section of the adrenal of Bradypus, the cortex is recognizable as a grayish zone inclosing a whitish medulla (fig. 7). In Choloepus, however, the cortex in the fresh exhibits a curious bright yellowish-orange color and possesses a hyaline-like transparency and consistency.

Frozen sections of the adrenals of Bradypus and Choloepus, stained with sudan III, show an ample amount of fat in the cortex, present in greatest quantity in a clearly defined zona glomerulosa.

Sections fixed in chromate solutions give a characteristic chromaffin reaction in the cells of the medulla. Macroscopic chromaffin bodies, so-called paraganglia, have not been observed on dissection of the retroperitoneal tissue after chromate fixation.

The kidneys[4]

Bradypus	Absolute weight of both kidneys, grams	Grams kidney per kilo total body weight	Grams kidney per kilo of corrected body weight
1	17 (adult)	3.63	4.38
2	19.6 (adult)	3.93	4.80
3	16.9 (adult)	3.92	4.81
4	10.05 (juvenile)	3.13	4.16
Choloepus			
1	23 (young adult)	5.59	6.66

[4] These observations can be compared with figures on kidney body-weight ratios given for other mammalian orders. The literature on this subject has been examined by Fox ('23), who gives tables on the normal ratios taken from his own observations, as well as those of others. Fox's tabulations for the kidneys are as follows:

Table showing weight of normal kidneys in relation to body weight. Number of specimens used to determine weight quoted in parenthesis

	Grams per kilo of body weight		Grams per kilo of body weight
Primates (5),	7.7	Ungulata (9),	3.5
Carnivora (6),	7.6	Edentata (1),	5.6
Rodentia (2),	15	Marsupialia (7),	7.6
Hyracoidea (1),	7.5	Monotremata (1),	11.2

The kidneys are situated low down in the abdominal cavity—a position which, taking into consideration their vascular supply, suggests to Hochstetter ('98) that they have undergone a descensus secondarily from a higher level. Hochstetter points out that the renal arteries arise from the aorta far anterior to the kidneys, taking a course almost parallel to the aorta for a long distance before reaching the kidneys. Similarly, the renal veins drain into the venae cavae some distance cranial to the upper poles of the kidneys.

In two fetuses in the present series (Bradypus, length, 49 mm.; Choloepus, 136 mm.) it has been ascertained that the kidneys are already pelvic in position. Moreover, the renal arteries at this period are given off from the aorta at the level of the upper pole of the kidneys, and not as in the adult far anteriorly to the kidneys. In view of this fact and that, as previously described, the adrenals are at first in contact with the kidneys, but subsequently in the adult come to lie far anterior to them, another explanation of the pelvic position and the blood supply of the kidneys suggests itself. It appears not unlikely that the pelvic position of the kidneys is a primary one, and not due to a descensus of the organs, and that the subsequent relationships of the long-drawn-out renal vessels to the kidneys is the result of a developmental shifting of the aorta and its tributary branches associated with the growth of the trunk. In this case the pelvis and the contained kidneys remain relatively fixed points, whereas the adrenals, as well as the aorta and its branches, undergo a shifting in their relative positions.

The female reproductive tract

The uterus of Bradypus and Choloepus, as well as of the other Xenarthra, is a simple, pear-shaped organ containing a single cavity. The fallopian tubes are coiled to either side of the uterus and open by slit-like orifices surrounded by a fringe of fimbria. The ovaries are concealed from view by

an ovarial pouch formed by a fold of mesosalpinx and the fimbriated end of the fallopian tube. The abdominal osteum of the tube and the orifice of the ovarial pouch are situated upon the dorsolateral wall of the uterus. When the ovarial pouch is opened, the ovary is disclosed. In both Bradypus and Choloepus, the ovary is a bilobed structure with a deeply cleft hilum (fig. 15). Contrary to the statement of Klinkowström ('95), the present observer has not found the ovaries inclosed in an ovarial pouch in the anteaters (Cyclopes, Tamandua). In the armadillo (Dasypus), on the other hand, the ovaries are inclosed as in the Bradypodidae. In contrast with those of the Bradypodidae, the ovaries of the anteaters and of the armadillo are fusiform.

The uterus of the sloths is a slender, pear-shaped organ flattened anteroposteriorly. It has been stated by some writers that the uterus of the sloth passes imperceptibly into the vagina, a cervical segment being absent. In the present material there seems no doubt that there is a specially differentiated segment between the uterus and vagina. The mucosa of the uterine cavity is smooth, but, as the slit-like cavity tapers toward the uterine orifice, its lumen becomes circular and the mucosa becomes thrown into a series of serrated folds in a zone 10 to 15 mm. in length which separates the uterine cavity from the vaginal cavity. The mucosa of the vagina, on the other hand, is characterized by the presence of ten to fifteen longitudinal folds. In keeping with statements in the literature, no such cervical segment has been found on dissection of the uterus of a specimen of Tamandua.

The vagina measures 15 to 30 mm. in length. It is stated in the literature of quite early date (von Baer, 1823; Forbes, '82; Klinkowström, '95, and many others) that the vagina of the Myrmecophagidae and Bradypodidae is peculiar in the possession of two narrow vaginal outlets instead of one. The writer's series of non-gravid uteri of sloths, although small, confirms this view and adds the additional fact that, besides being double, the vaginal orifices are com-

pletely closed during some periods by a vaginal closure membrane.

On dissecting the vagina of the sloth, it is discovered that only the lower third is double, a wedge or septum of tissue projecting from below into the vaginal cavity producing two funnel-shaped vaginal canals. The writer had some difficulty in ascertaining by dissection of fixed material collected in 1925 whether these canals are patent or not, the belief expressed (Wislocki, '27) being that in several specimens they did not communicate with the urogenital space. During the summer of 1927 an opportunity arose to test this upon fresh specimens. The method adopted was to inject India ink into the uterus, thereby filling the uterus and vagina, and to watch for its escape into the urogenital canal. By this means it was ascertained that the vagina at some periods lacks all communication with the exterior. Unfortunately, the number of non-pregnant sloths was so small this year that the relationship of the closure of the vagina to the reproductive cycle could not be ascertained. The results were as follows: In four pregnant specimens of Bradypus, ink injected under some pressure did not escape to the outside, but merely ballooned out the vagina into an upper simple tubular segment which communicated below with two short cornua which ended in the wall of the urogenital sinus without any escape of fluid. Under still greater pressure the ink extravasated into the tissue at the tips of the vaginal canals filling a plexus of lymphatics in the vaginal closure membrane. In two non-gravid specimens of Bradypus the experiment was also tried. In one, as in the gravid specimens, the vaginal canals were found closed. In the other specimen the vaginal orifices were found open, the ink emerging as two narrow jets into the urogenital sinus to the right and left of the midline. The vaginal outlets were no more than 1 to 2 mm. in greatest diameter. In the single female specimen of Choloepus obtained, the vagina was found closed. A slight difference existed, however, between it and the three-toed sloth in that the two vaginal canals were much closer together

in Choloepus; hence the median septum was much thinner
than in Bradypus and the apices of the cornua in the wall of
the urogenital sinus were much closer together.

The ovaries of these two specimens of Bradypus and the
one of Choloepus were sectioned serially. The ovaries of
Bradypus and Choloepus, in which the vaginal orifices were
closed, proved on section to be quiescent ovaries in which
there are neither maturing follicles nor recent corpora lutea.
Besides scores of primary follicles near the surface of the
ovary, there is a group of graafian follicles, situated in the
deeper portions of the ovary, the largest of which is not over
a half of a millimeter in diameter.

The ovaries of the specimen of Bradypus, in which the
vaginal orifices were open, also contain only immature fol-
licles. In the left ovary, however, there is a mass some
2 mm. in diameter buried in the ovary which proves to be a
corpus luteum. It is composed of a rim or cortex of typical
lutein cells and stroma, surrounding a cavity containing a
non-cellular mass which appears to consist, in part of
fibrinous, in other part of eosin-staining, collagenous ma-
terial. A few deeply pigmented wandering cells are scattered
throughout this central core.

Further details on the reproductive tracts of sloths may be
found in the papers by Klinkowström ('95) and Wislocki
('27).

In the present series opportunity was given to study gesta-
tion and placentation in Bradypus griseus, a detailed account
of which is given in another place (Wislocki, '27). However,
the writer has recently obtained a fetus and placenta of
Choloepus hoffmanni, only one other placenta of which has
been described heretofore (Turner, '73). In the following
passages a description of this specimen will be given, as well
as a short summary of the placentation of the three-toed
sloth.

Out of six female specimens of Choloepus autopsied at
Barro Colorado Island during June, July, and August none
was found to be pregnant. One specimen of Choloepus, kept

captive for several months, aborted a dead fetus in the month of October. This fetus, judging from its development, is from about the midgestational period. However, of forty mature female specimens of Bradypus griseus which have come to autopsy over a period of three years during the months from April 12th to September 1st, thirty-one were pregnant. No early embryonic stages have been obtained so far, the youngest fetuses being 27, 36, and 47 mm. (length of spine without tail), respectively. In general, it may be stated that the oldest fetuses have been obtained during the late summer months.

The fetus and placenta of Choloepus hoffmanni which the writer obtained recently were in an excellent state of preservation, and hence the placenta could be studied. The fetus measures 136 mm. length of spine without tail, and is without hair or an epitrichial membrane which had yet separated. The placenta is in the gross essentially like the placenta of the three-toed sloth at the same period of gestation. It consists of irregularly distributed lobules of placental tissue covering much of the chorion, with membranous chorion between the nodules. Each lobule varies in size and thickness from 1 to 1½ cm. in diameter and 2 to 5 mm. in thickness. These lobules in the fresh specimen are reddish in color. Besides these lobules, there is a smaller number of nodules which are not so large and are of a white or greenish color, in the fresh state. These are degenerating lobules and are identical with those observed in the placenta of Bradypus. Besides these, there are thin brownish placques in the chorion, the final state of degenerated lobules before their complete disappearance. The placenta is seen to be composed of diffusely scattered nodules and differs from the placenta of Bradypus at this period of gestation in not showing a tendency of the chorion to become completely membranous at the cervical pole. Turner's specimen from the end of gestation is described as bell-shaped, occupying the fundic pole of the uterus. It is quite likely, from Turner's specimen and our knowledge of the metamorphosis of the placenta in

Bradypus, that the placenta in the present case would have eventually become localized in the fundic half of the uterus by further degeneration of lobules. The minute amniotic caruncles or villi which the author has described for the placenta of Bradypus are lacking in this specimen. The umbilical cord is smooth, as in Bradypus; its attachment and the distribution of the blood vessels supplying the placental lobules are identical with the conditions observed in the three-toed sloth.

Microscopically, the placenta appears to be identical with that of Bradypus, being deciduate, each placental lobule consisting of a labyrinth of chorionic lamellae inclosing maternal vessels lined by endothelium and an associated supporting reticulum upon which the endothelial cells rest.

From the present series the following observations on the placentation of Bradypus griseus are given in summary: Only one young matures at a time. One corpus luteum develops in either right or left ovary. The corpus luteum occupies one pole of the enlarged bean-shaped ovary and does not protrude (fig. 26).

The chorion is invasive and in the youngest stages of this series consists of small lobules diffusely attached to the uterine wall. As the uterine cavity enlarges, the lobules increase in size. However, with advancing gestation, the placenta undergoes a marked change in shape, ceasing to be diffuse and becoming localized. This is brought about by a degeneration of a large portion of the original lobules at the cervical pole of the uterus, so that the placenta becomes localized finally in the fundic half of the uterus as a single or bilobed lobulated mass. The remainder of the chorion thereby eventually becomes membranous. Minute amniotic caruncles are abundantly present over the amnion, which at an early date fuses completely with the chorion, thus obliterating the exocoelome. A small yolk sac is present during the early period before the exocoelomic cavity is obliterated.

Microscopically, the placenta of Bradypus is found to be of the deciduate type. Each lobule is found to be composed of tortuous lamellae of chorionic ectoderm (trophoblast) which inclose maternal sinuses within them. The maternal sinuses are lined by endothelial cells which rest upon a delicate basement membrane, the latter the remains of uterine stroma. These maternal blood vessels are inclosed within lamellae of chorionic syncytium. The lamellae of chorionic ectoderm rest upon central cores of fetal connective tissue in which the fetal blood circulates. A placenta of this microscopic nature represents a transition form from the placenta syndesmochorialis to the placenta endotheliochorialis, according to the classification of Grosser. In its microscopic structure the sloth's placenta possesses many points of similarity to that of Carnivora.

The preceding descriptions and discussions have brought out that the placentae of the didactyl and tridactyl sloths resemble one another very closely. The placenta of the anteaters has been described variously in brief observations as a thick roundish cake (Mayer, Myrmecophaga), fungiform (Welcker, Myrmecophaga), unilobed and dome-like (Milne-Edwards, Tamandua), and as disk-like or dome-shaped (Ryder, Cycloturus). The present writer has recently obtained a gravid uterus of Cyclopes (fetus measuring 92 mm. from snout to base of tail) in which the placenta occupies the fundus of the uterus as a discoidal, superficially lobulated mass. In the fetus belonging to this specimen as well as in a nearly mature fetus of Tamandua, the author has not observed the presence of an epitrichial membrane.

Microscopically, the placenta of Cyclopes is entirely different from that of the Bradypodidae. In the present specimen it is found to be of a labyrinthine type with wide maternal lacunae surrounding stout cords of fetal tissue which have a resemblance to villi. It is very likely of the hemochorial type, according to Grosser's classification, although final judgment on this point and a full description of the specimen will be reserved for a future communication.

Placentation in the Dasypodidae, the remaining family of the Xenarthra, is markedly modified by the condition of polyembryony. The placenta undergoes marked changes in gross form during the course of gestation and exhibits, therefore, a superficial resemblance to the sloth's placenta. However, microscopically, the placenta of the armadillo shows no resemblance to that of sloths, being composed of short, stout villi surrounded by maternal lacunae which constitute an intervillous space. The microscopic structure of the placenta of Dasypus appears to resemble that of Cyclopes, as far as an opinion can be based upon our present fragmentary knowledge of the microscopic structure of the placenta in these two forms.

Some characters of fetuses of the tridactyl sloth have been described elsewhere (Wislocki, '27). One of the most interesting is the presence of an epitrichium—a cornified layer of the epidermis which separates from the body of the fetus as a continuous sheet or membrane as the hair erupts. The formation of the free epitrichial membrane occurs at the 130- to 150-mm. stage. The membrane resembles superficially an amnion inclosing the fetus, but it differs from the latter in that it remains attached to the epidermis around the eyes, nostrils, and other body orifices. In a fetus of Tamandua near term, as well as in a mature fetus of Cyclopes, possessing hair, the writer has not observed an epitrichial membrane.

The young of Bradypus are born in a very mature state. A newborn measured 31 cm. from snout to tip of tail. The hair coat is well developed, many of the hairs being over a centimeter long. The newborn are able to use their arms and legs effectively in clinging to the fur of the mother. The eyes are open at birth. The mother has two pectoral nipples. According to Beebe ('26), who describes the development and behavior of a newborn, the young are weaned at the end of four to five weeks.

Differences exist in the structure and form of the pelvis in adult male and female specimens of Bradypus (fig. 12).

The pelvic outlet is relatively larger in the female and the bones are more slender than in the male. The pubic bones, especially in the female, are extremely slender. The symphysis pubis forms in the adult a bony union which remains united throughout pregnancy. In juvenile specimens there exists a separate interpubic bone in the symphysis which finally unites with the pubic bones to form a complete bony symphysis.

As has been stated, the author has secured one fetus of the didactyl sloth, Choloepus hoffmanni (figs. 3, 4, and 5). This fetus is 136 mm. long (length of spine) and 200 mm. from the tip of the snout to the rump. Fore and hind limbs are nearly of equal length, as in the adult, whereas in Bradypus the hind limbs are much shorter than the fore limbs in prenatal, as well as postnatal life (figs. 1 and 2). The neck is short, instead of long as in Bradypus, and the head is not flexed upon the chest. The external ears are well developed, the eyes and nostrils closed. Around the mouth on the upper and lower lips there are several rows of cutaneous papillae, from the tip of each of which a hair or vibrissa protrudes. The length of these vibrissae is about 1 mm. They are also encountered in Bradypus, appearing first in 70- to 80-mm. fetuses, and reaching a length of 2 or 3 mm. toward the end of gestation. After birth these vibrissae disappear, the snout becoming smooth and hairless.

Aside from the vibrissae protruding from the cutaneous appendages about the mouth, the Choloepus fetus at this stage is hairless, differing from the fetus of Bradypus in which the hairs begin to erupt rapidly at the 130- to 135-mm. stage. In the specimen of Choloepus the hair follicles in the skin are just barely visible to the naked eye. There is not the slightest indication of the separation of the epitrichium, whereas in Bradypus of comparable size the epitrichial membrane is well separated. Choloepus develops a free epitrichial membrane later, as is clear from Turner's description of a fetus of the didactyl sloth near term which possessed a complete epitrichial membrane. Furthermore, in

the fetus of Choloepus the skin is not pigmented in the same manner as in Bradypus. The pigment imparts a scarcely perceptible grayish tint to the entire epidermis, with the exception of the palm and sole pads, while over the dorsum, shoulders, crown of the head, and on the eyelids a brownish element in the pigmentation is visible. In the fetuses of Bradypus, on the other hand, a mottled, black pigmentation of the head and trunk is conspicuous.

Male reproductive tract

Original observations upon the male reproductive tract of the Bradypodidae exist in the works of Meckel (Choloepus, 1811), Rapp (Choloepus, '52), Oudemans (Bradypus, '92), Klinkowström (Bradypus, '95), van den Broek (Bradypus, '12), and Kaudern (Bradypus, '14). Except for the observations of Meckel and Rapp, the descriptions, especially the recent ones, are devoted to specimens of Bradypus alone. The present writer has made no attempt to study the male reproductive tract intensively. The subject would require thorough knowledge and study of the comparative anatomy of the male reproductive tract in Mammalia in general, more especially in the complex field of the development and microscopic anatomy of the reproductive tract. Nevertheless, several important observations have been made on the present material which will be included in a short summary of the existing knowledge of the male reproductive tract in the Bradypodidae.

The testes of the Bradypodidae are intra-abdominal, lying in the pelvis between the bladder and the rectum each in a duplication of the peritoneum, the urogenital mesentery, a conspicuous band which extends from the testes upward to the adrenal body. An inguinal canal and inguinal ligament are lacking (Weber, '98, '04). According to van den Broek ('12), the testes of Bradypus undergo a partial or rudimentary descensus whereby they attain a pelvic position. A rudimentary inguinal ligament is present during the period of descent of the testis, but subsequently disappears. The

testes are spherical. The epididymis lies medial to the testis and the two epididymides are united across the midline by a strong band of connective tissue.

It is of interest to note that there may be a correlation between the intra-abdominal location of the testes and the body temperature of the sloth. As Richter ('23, unpublished), Ozorio de Almeida and Branca de A. Fialho ('24), and Kredel ('27) have observed, the body temperature of the sloth is low and the temperature-regulating mechanism poorly developed. Recently, the author has observed that in the armadillo, which also possesses intra-abdominal testes, the body temperature is low and fluctuates considerably with the environmental temperature. Moore ('26) has shown that spermatogenesis requires lower temperatures than those prevailing in the body cavity of mammals possessing descended testes and that return of the testes periodically to the abdomen, normally or by instrumental means, effectively suppresses spermatogenesis. It appears not unlikely, then, that mammals possessing abdominal testes throughout life have lower body temperatures, as in the case of the sloth and armadillo, so that descensus is unnecessary in these forms to insure the maturation of spermatozoa.

Microscopically, the testes of Bradypus and Choloepus in the present series show active spermatogenesis within the seminiferous tubules. Interstitial tissue is abundant in the sloth's testis. However, it is greater in amount in Choloepus than in Bradypus and is characterized in the former by the presence of large, polygonal cells which contain an abundance of yellowish pigment within their cytoplasm (fig. 28).

Careful dissections have been made of the urogenital passages and accessory reproductive glands in Bradypus by Klinkowström ('95) and Kaudern ('14). A prostate gland inclosed in the urethral muscle, rudimentary seminal vesicles, and Cowper's glands have been described by Klinkowström. The uterus masculinus is small; the penis, cleft. The prostate gland, according to Kaudern, is represented by a large mass of glandular tissue which lies between the neck of the bladder

and the openings of the vasa deferentia, inclosed within the urethral musculature, instead of outside of it, as is the usual arrangement of the mammalian prostate. In the young animal which Kaudern dissected he was unable to find the rudimentary seminal vesicles described by Klinkowström. Oudemans ('92) also states that the seminal vesicles are lacking in Bradypus.

Concerning Choloepus, less is known. Its testes are described as being abdominal, Cowper's glands present, while the presence of a prostate and seminal vesicles is disputed. According to Rapp ('52), in Choloepus didactylus the seminal vesicles are very large, and consist of thick, much-coiled tubes. However, Meckel (1811) convinced himself that there are no seminal vesicles in Choloepus. Klinkowström chooses to consider the statement of Meckel more reliable than that of Rapp.

The present series contains ample material of the male reproductive tracts of both Choloepus and Bradypus. Dissection of these specimens reveals the presence of extremely large seminal vesicles in Choloepus, of rudimentary ones in Bradypus. The description of these specimens follows: The testes of Choloepus and Bradypus are very similar in appearance. The testis proper is spherical; the epididymis lies medial to the testis. The caudal ends of the epididymides are united across the midline by a dense band of connective tissue. This fibrous union is denser in the two-toed than in the three-toed sloth. In both forms the vasa deferentia consist of compact, serpentine coils closely united by connective tissue. The seminal vesicles differ markedly in the two forms. In Choloepus two long tubes, some 10 cm. long when straightened out and about 1½ to 2 mm. in diameter, can be dissected out from the connective tissue near the base of the bladder. In situ these vesicles are compactly coiled and embedded in connective tissue. The seminal vesicles open independently of the vasa deferentia into the urogenital sinus. In Bradypus, on the other hand, the seminal vesicles are short, round, slightly twisted tubes. In adult specimens they range from

½ to 1 cm. in length and 2 mm. in diameter. They drain dorsally into the urogenital sinus in the neighborhood of the prostate about 1 cm. from the openings of the vasa deferentia. The prostate is similar in both forms, consisting of a marked glandular swelling surrounding the urethra immediately adjacent to the neck of the bladder.

The microscopic appearances of these glands will not be described, but several photographs of their microscopic structure are shown (figs. 27 and 29).

The writer has not had an opportunity to dissect the male reproductive tract of any of the anteaters. However, three male specimens of Dasypus have been autopsied. They possess a well-developed bilobed prostate outside of the urethral musculature and large, stout, vesicular seminal vesicles. Klinkowström was in doubt as to the presence of seminal vesicles in Dasypus.

THE THORACIC VISCERA

The heart

Bradypus	Absolute weight of heart, grams	Grams heart per kilo of total body weight	Grams heart per kilo corrected body weight
1	10.9 (adult)	2.34	2.82
2	11.5 (adult)	2.67	3.29
3	12.5 (adult)	2.9	3.55
4	7.5 (juvenile)	2.4	3.08
Choloepus			
1	14.6 (young adult)	3.54	4.25

Figures taken for comparison from the compilations of Fox ('23) are: Weight of normal heart in relation to body weight. Number of specimens used to determine weight quoted in parenthesis.

	Grams per kilogram of body
Man (4),	5.67
Primates (4),	6.56
Carnivora (6),	6.78
Rodentia (5),	5
Ungulata (10),	5.8
Marsupialia (3),	5.1

The lungs

The lungs of Bradypus and Choloepus possess no fissures separating the lobes, the pleura passing directly from lobe to lobe without dipping into incisurae or forming any surface irregularities which would indicate the presence of lobes. Nevertheless, in a fetus of Bradypus of 120 mm. length, the lungs are found to consist of numerous primary lobules, the separation of which by connective tissue can be made out by the naked eye. There is also clearly present a demarcation of the lungs, right and left, into two lobes, an upper and a lower one, separated not by a cleft, but by loose connective tissue which the pleura bridges at the surface. Hence it appears that the lungs are established in the embryonic period with distinct lobes, united by loose connective tissue, the boundaries of which become practically obliterated in the postnatal lungs, so that in the adult neither lobes nor fissures are visible on the surface. Furthermore, the pleural covering is unusual, as Weber ('04) points out, in that, anteriorly in the region of the pericardium, the pleura is not reflected onto the pericardial surface of the lung to extend to the hilum, but is reflected directly from the mediastinal pulmonary border to the wall of the thorax. Thus the pericardial connective tissue and the capsule of the lung are firmly united without the interposition of serous membrane (Weber, '04). The pleura covering the lungs is extremely delicate in the sloth. Microscopically, nothing noteworthy has been observed in the structure or arrangement of the pulmonary alveoli.

The tracheas of Bradypus and Choloepus are very dissimilar. As Weber ('04) describes and pictures, the trachea of Bradypus is very unusual. It is extremely long. It extends along the vertebral column to the diaphragm, where it turns sharply upon itself, forming a complete U. It then extends upward to the hilum of the lungs, where it bends ventrally and turns upon itself again, forming an inverted Y this time, as it divides into right and left bronchi (fig. 9). In two adult specimens which were measured, the lengths from

the thyroid cartilage to the turn at the diaphragm were 21 and 23 cm., respectively; the distances from this turn to the second one at the hilum of the lungs, 4 and 5 cm., respectively, giving total lengths of 25 and 28 cm.

In Choloepus, on the other hand, the trachea is extremely short, dividing immediately after its entry into the thorax, high up in the mediastinum, into bronchi which enter at once into the lungs. In two specimens measured, the lengths of the trachea to its bifurcation were 9 and 10.5 cm., respectively.

As Weber has observed, the curious form of the trachea in Bradypus is already present in fetal life. He says that the morphological and physiological significance of the structure is a complete puzzle.

However, the opportunity to compare Bradypus with Choloepus in the present study suggests a possible explanation. The neck of Bradypus is extremely long, that of Choloepus short; Bradypus normally possesses eight or nine cervical vertebrae, Choloepus only six. Furthermore, as Beebe ('26) has pointed out, Bradypus cuculliger possesses a surprising mobility of the neck. In the sagittal plane the head is capable of movement anteroposteriorly through an arc of 270°, while it can be rotated through three-quarters of a circle—an extraordinary degree of rotation for a mammal. The same is true of Bradypus griseus, so that such mobility seems to be a characteristic of the three-toed sloths. Choloepus, on the other hand, with its short neck, has a very limited range of movement of the head, scarcely half the amount of Bradypus. Thus, it seems not unlikely that the long trachea in Bradypus is correlated with the enormous range of movement of the neck and head in this form, the thoracic tracheal loop serving as slack which can be utilized when necessary. This idea is strengthened by actual observations on the cadavers of freshly killed three-toed sloths, with the thorax opened, so that the trachea could be observed. In such a preparation with the neck extended, when the head was grasped and rotated, the tracheal loop could be observed to diminish in length by 2 or 3 cm.

The comparison of the lungs of Dasypus, Tamandua, and Cyclopes with those of the Bradypodidae is of interest. The trachea in the anteaters and armadillos is short as in Choloepus. The lungs themselves in Dasypus are completely separated into lobes by deep fissures. In Tamandua and Cyclopes, however, the lobes are demarcated by superficial fissures only, while in the Bradypodidae, as has been described, there is no separation into lobes visible on the surface.

The thymus occupies the anterior mediastinum as a compact lobulated mass. Microscopically, in young adult animals it presents large lobules with poorly separated cortical and medullary zones and conspicuous Hassall's corpuscles (fig. 33).

THYROID

Bradypus	Absolute weight of thyroids, grams	Grams thyroid per kilo of total body weight	Grams thyroid per kilo corrected body weight
1	0.5 (adult)	0.107	0.129
2	0.45 (adult)	0.123	0.157
3	0.65 (adult)	0.150	0.182
4	0.4 (adult)	0.079	0.097
5	0.38 (adult)	0.088	0.135
6	0.2 (juvenile)	0.064	0.073
Choloepus			
1	0.25 (young adult)	0.061	0.073

The amount of thyroid tissue per kilo body weight compared with the total body weight is extraordinarily low. Even the figures obtained by comparison with a corrected body weight (body weight, minus stomach contents and urine) are the smallest proportional weights known for any mammal, if the measurements given by Fox ('23) are consulted. The latter's figures were obtained from twenty animals with apparently normal thyroids. The lowest weights were found in the ungulates which is the only group which approaches the sloth in relative smallness of the thyroid glands.

The thyroid tissue of Bradypus and Choloepus is composed of two independent glands, one to either side of the thyroid cartilage. The glands are spindle-shaped and mucoid or gelatinous in texture. That of Bradypus is pink in color, whereas that of Choloepus is strikingly orange in color.

On microscopic section, the glands are found to be composed of small follicles containing colloid. The gland is poorly defined, the groups of follicles being irregularly interspersed between large amounts of fat. The contour of the gland is extremely irregular and a capsule is lacking (fig. 30). Colloid is present in the majority of the follicles in amounts which by comparison with other mammalian thyroids might be considered to be within the range of normal. There is certainly no accentuation of the amount of colloid. Although the colloid is fairly abundant in most of the lobules, there are numerous areas in which the colloid is decreased in amount, the follicles being small with a relatively high epithelium. Finally, areas of transition exist from those of relatively depleted follicles to areas in which the tissue is solidly epithelial in character with only an occasional small follicle containing a droplet of colloid.

The epithelium of the colloid-containing follicles, which form the bulk of the glands, is usually cuboidal, although slightly higher in some follicles and lower than cuboidal in a few follicles. The nuclei are invariably round. Free cytoplasm is usually exhibited at either pole of the cells. The cytoplasm at the distal pole of the cells occasionally contains chromophobe colloid droplets. The colloid within the follicles stains variably with Mallory's connective-tissue stain, the majority of the follicles containing blue-staining colloid; a smaller number, red-staining colloid. Besides the stainable colloid, these follicles almost invariably contain, in the periphery of the inclosed colloid in contact with the epithelium, numerous droplets of chromophobe colloid which appear in section as rows of unstained vacuoles (fig. 32). From the presence of the chromophobe secretion within the lumen of the follicles, as well as similar droplets within the distal cytoplasm of the epithelium, and from the relative height of the epithelial cells and the fact that their nuclei are round, one is forced to conclude that the thyroids are in a state of active secretion.

Parathyroid tissue has been observed in a number of the sections of thyroids. Whether there are four parathyroids present in the sloth and, if so, where they are located has not been observed. The parathyroid tissue encountered in the sections of thyroid presents nothing worthy of special description. In one specimen a mass of thymic tissue has been found in association with the thyroid.

HYPOPHYSIS

Nothing unusually noteworthy has been observed concerning the hypophysis. Two glands, of both Bradypus and Choloepus, have been sectioned serially and stained in haematoxylin and eosin and by Mallory's connective-tissue stain.

The hypophysis of Bradypus is rather singular in shape. It rests in a shallow sella turcica and is flattened out into an ovoid structure some $5\frac{1}{2}$ by 4 mm. in diameter and only 2 mm. thick. An infundibulum attaching anteriorly ends in a round posterior lobe which is inclosed by the flattened hood-like pars anterior excepting its dorsal surface. The hypophysis of Choloepus is nearly spherical.

Microscopically, the only unusual feature of the hypophysis noted is the abundant development of small cysts at the junction of the epithelial and neural portions of the gland and the projection of these cysts or similar gland-like structures from the junctional zone far into the region of the posterior lobe (fig. 31). Many of these cysts contain free mononuclear cells, besides a finely granular secretion within their lumina.

BRAIN

No attempt has been made to study the brains of either of the two sloths. The gross features of the brains of the Xenarthra, including specimens from the sloth, have been studied by Pouchet ('96) and by G. Elliot Smith ('99). The latter points out besides certain primitive characters, a rather striking similarity of the pallium to that of carnivores.

In the present investigations, examination of the retina, fixed in Held's fluid according to the modification of Detwiler, reveals that the retina of sloths is composed exclusively of rods.

DISCUSSION AND CONCLUSIONS

Should the present study prove of value, it is mainly because it attempts to compare the tridactyl and didactyl sloths, using particularly the morphology of the viscera and certain other of the soft parts as a basis for comparison. Many of the findings are new; others of them are, however, a recapitulation of older observations. Moreover, the study presents some data, derived from the dissection of the soft parts of several members of the Myrmecophagidae and Dasypodidae, which offer a basis for a comparison and a discussion of the relative positions of the sloths to the other members of the Xenarthra. This comparison, it is believed, has been especially valuable in the study of the vascular plexuses of the extremities and of the transformation of the gall bladder in the Xenarthra.

A tabulation of the principal points of similarity and of dissimilarity, discussed in this paper, between the three-toed and two-toed sloths, will first be given. Following that, the data derived from a comparison of the entire group of the Xenarthra will be summarized.

The following points of similarity between the tridactyl and didactyl sloths were noted: striated musculature uniformly red throughout; presence of highly specialized vascular structures, the so-called vascular bundles, in the extremities; venous anastomoses between the vena cava and the epidural veins whereby much of the blood is returned to the heart; lymphatics emptying in the adult directly into the abdominal veins with a suppression of the thoracic duct; complex stomach adapted to a herbivorous diet; rectal pouch; rotation of the liver; large pancreas; pelvic kidneys; intra-abdominal testes; prostate in urethral wall; bilobed ovaries, contained in ovarial pouches; uterus simplex; a cervix

between uterus and vagina; vagina double in lower portion and closed at times by a vaginal closure membrane; placenta deciduate of the endotheliochorial type, lobulated and undergoing a characteristic change in shape during gestation; invariably one young; an epitrichial membrane investing the fetus; adrenals, thymus, thyroids, and hypophysis grossly and microscopically similar; lack in lungs of surface demarcation into lobes, due to absence of fissures; thick skin, due to dense fibrous character of the corium; absence of subcutaneous adipose tissue.

The following are the main points of dissimilarity noted in the two forms: trachea peculiarly modified in Bradypus; absence of gall bladder in Bradypus, presence of a rudimentary one in Choloepus; presence of large anal glands in Choloepus; rudimentary seminal vesicles in Bradypus, large seminal vesicles in Choloepus; presence of large, pigmented interstitial cells in the testes of Choloepus; spleen slender, long, and round in Bradypus, triangular and flat in Choloepus; relative proportions of anterior and posterior extremities markedly different in the two species, associated during life with entirely different postures and manner of climbing in the two forms.

The morphological differences in the skeletons of the extremities of the two forms of sloths have been known to anatomists for years, but that they are correlated with entirely different postures and modes of climbing in the two genera, as has been determined here by the observation of abundant living specimens, is quite significant. These observations not only emphasize the wide separation of the two genera, but influence all teleological reasoning concerning the development of certain characteristics of the two types of sloths. The doctrine of adaptation to purpose is useful only when it is based on sound morphological, as well as physiological, observations. Much of the previous reasoning relating to causes was founded upon the assumption that the hanging postures are identical in the two forms of sloths. Since this is not correct, many of the older explanations

become obsolete and new teleological principles must be sought, depending upon the observed differences in posture in the three-toed and two-toed sloths.

By including representatives of the entire group of Xenarthra within the investigation, much new light has been shed upon the relationships of this group. Perhaps the most interesting of these relationships is the correlation which has been brought out between the character of the vascular plexuses found in the extremities of the Xenarthra and their individual postures and muscular activity. This correlation begins to bring order into the much-vexed question of the nature and significance of the vascular plexuses in the extremities. It suggests that the vascular bundles of the sloths and of Cyclopes are organs sui generis, associated with highly specialized functional and morphological developments in the limbs of these animals. Moreover, they are derived possibly from simpler ancestral vascular plexuses of the character still present in other members of the existing Xenarthra, such as Dasypus and Myrmecophaga. The diversity of the plexuses in the different groups makes improbable the explanation that they all represent simply the persistence of an indifferent type of embryonic vascular network. They appear in the sloths to be more likely highly specialized derivatives of simple embryonic patterns. The observations upon this correlation are summarized in the following table.

Species	*Pattern of vessels of extremities*	*Mode of life, muscular activity*
Dasypus	Simple plexiform networks in the forearms and legs. Striated musculature, mixed red and white	Ground-living. Extremely active. Repeated, rapid movements in digging over prolonged periods
Myrmecophaga	Simple plexiform networks in the forearms and legs. Character of striated musculature not known	Ground-living. Active in walking
Tamandua	More complex plexiform networks in upper arms and thighs, as well as forearms and legs. Character of striated musculature not known	Largely arboreal. Less active than either of the preceding. Its movements are to some extent deliberate and slow like those of sloths

Species	Pattern of vessels of extremities	Mode of life, muscular activity
Cyclopes	Vascular bundles highly differentiated in arms and thighs. Character of striated musculature not known	Arboreal. Movements slow and deliberate. Clinging postures, necessitating highly developed postural tone, maintained for long periods
Choloepus and Bradypus	Vascular bundles highly differentiated in arms and thighs. Striated musculature uniformly dark red	Arboreal. Movements slow and deliberate. Adaptation to clinging with maintenance of postural tone highly developed

The morphology of the gall bladder constitutes another interesting object for comparison within the order of the Xenarthra. It has been found in the present investigation that the gall bladder is absent in Bradypus, rudimentary in Choloepus, only slightly more highly developed in Cyclopes and Tamandua, respectively, while in Dasypus it is large and complex.

The trachea and lungs in the Xenarthra also exhibit noteworthy modifications which parallel the relationships between the genera brought out in other characters. The trachea is markedly modified in Bradypus, whereas in the remaining Xenarthra it is of the usual mammalian type. In reference to the character of the trachea, as in the instance of the gall bladder, Bradypus is further removed than Choloepus from the remaining members of the group. Furthermore, in Bradypus and Choloepus the lobes of the lungs are firmly united by connective tissue, so that there are no surface indications of lobulation, the pleura clothing the entire surface of the lungs uninterrupted by fissures. In Tamandua and Cyclopes the lungs are superficially divided into lobes, and in Dasypus the demarcation of the lobes by fissures is complete.

Lastly, in regard to the reproductive tract, so many observations which differ from previous ones in the literature have been made in this investigation that it is deemed admissible to summarize the data on this subject (table 1).

The following new observations upon the reproductive tracts of the Xenarthra are worthy of recapitulation:

1. A cervical segment is present in the Bradypodidae, between uterus and vagina, consisting of a region of especially modified mucosa.

TABLE 1

	BRADYPUS	CHOLOEPUS	TAMANDUA	DASYPUS
Uterus	Simple	Simple	Simple	Simple
Cervix	Present	Present	Absent	Absent
Septum vaginae	Present	Present	Present	Absent
Vaginal closure membrane	Present	Present	No observations	No observations
Ovarial pouch	Nearly complete	Nearly complete	None	Nearly complete
Ovary	Bilobed	Bilobed	Fusiform	Fusiform
Placenta	Lobulated and consisting microscopically of chorionic lamellae inclosing maternal vessels. Endotheliochorial type. One young	Lobulated and consisting microscopically of chorionic lamellae inclosing maternal vessels. Endotheliochorial type. One young	Discoidal in Tamandua and Cyclopes. In Cyclopes consists of a labyrinth of maternal lacunae surrounding fetal villi. Probably hemochorial	Segmented in correlation to polyembryony. Consists microscopically of maternal lacunae surrounding fetal villi. Probably hemochorial
Epitrichial membrane	Present	Present	Absent	Absent
Testes	Intra-abdominal. Interrenal position	Intra-abdominal. Interrenal position	Intra-abdominal	Intra-abdominal. Anterior body wall at inguinal ring
Prostate	Present in urethral muscle	Present in urethral muscle	No observations	Present, bilobed, outside of urethral muscle
Seminal vesicles	Rudimentary	Large	No observations	Large

2. Besides the presence of a vaginal septum, which was known to the older observers, a vaginal closure membrane of periodic occurrence has been found in the Bradypodidae.

3. The ovaries of the Bradypodidae and Dasypodidae are contained within an ovarial pouch which has a narrow, slit-like orifice into the peritoneal cavity. In Tamandua the ovaries are not contained in a pouch.

4. The placentae of the Bradypodidae, Myrmecophagidae, and Dasypodidae differ materially in gross and microscopic character. The placentae of Bradypus and Choloepus are almost identical, consisting of diffuse, lobulated, deciduate masses which gradually become localized placental masses during the course of gestation. According to the author's interpretation, the placenta of the Bradypodidae belongs to the endotheliochorial type of Grosser.

The placenta of a specimen of Cyclopes which the author describes is a localized discoidal placenta, agreeing with several older observations in the literature on the form of the placenta in the Myrmecophagidae. Microscopically, it consists of short, stout strands or villi of fetal tissue surrounded by maternal lacunae. Hence it is totally unlike the placenta of the Bradypodidae. It is deciduate and is probably of the hemochorial type of Grosser.

In the Dasypodidae the placenta is adapted to the condition of polyembryony and differs markedly in gross character from either of the two previous forms. Microscopically, it is villous in character and possesses wide intervillous maternal lacunae, bearing considerable resemblance to the placenta of Cyclopes. It is deciduate and is probably of the hemochorial type of Grosser.

5. The fetuses of the tridactyl and didactyl sloth differ markedly in external form and body proportions. Moreover, they differ in the time at which the hair erupts and a free epitrichial membrane develops. In Tamandua a free epitrichial membrane was not observed in a specimen near term, possessing long hair.

6. The testes of the Xenarthra are intra-abdominal. It is suggested in the present study from observations on sloths and armadillos that there is a correlation between intra-abdominally located testes and body temperature.

7. A prostate gland inclosed in the urethral musculature has·been found in both Bradypus and Choloepus. In Dasypus the prostate is bilobed and lies outside the urethral musculature.

8. In disagreement with previous observations, large seminal vesicles have been found in Choloepus, rudimentary ones in Bradypus. In Dasypus seminal vesicles are present and well developed.

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PLATES
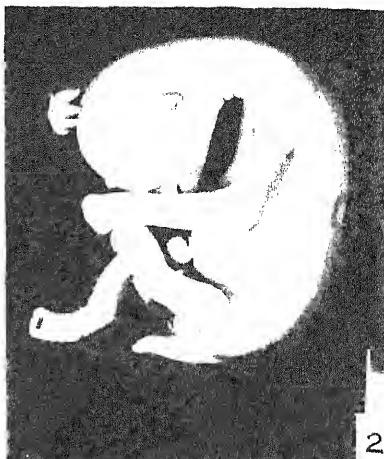
PLATE 1

1 Fetus of Bradypus griseus griseus. Length of fetus: spine without tail, 97 mm.; snout to tip of tail, 130 mm. This photograph shows the characteristic posture of the fetus in utero. It also shows the great disproportion of the anterior and posterior extremities in Bradypus, as well as the unusual length of the neck. Hair has not erupted yet, nor has an epitrichial membrane formed. $\times$ 1.4.

2 Same fetus as in figure 1, seen from opposite side. $\times$ 1.4.

3 Fetus of Choloepus hoffmanni. Length of fetus: spine without tail, 136 mm.; snout to rump, 200 mm. In Choloepus the anterior and posterior extremities are nearly of equal length. The neck is short and stout. Around the mouth the vibrissae can be seen. Hair has not erupted yet at this stage in Choloepus, nor has the epitrichial membrane separated. $\times$ 0.7.

4 Ventral view of same specimen as in figure 3. $\times$ 0.7.

5 Opposite side of same specimen as in figure 3. $\times$ 0.7.

PLATE 2

6 Bradypus griseus griseus. Section through the vertebral column of a nearly term fetus, showing the vertebra, the vertebral canal, the spinal cord, and on the right side the epidural vein, fully as large as the spinal cord, which represents a principal channel in sloths for the return of blood from the caudal part of the body to the heart. Mallory's connective-tissue stain. × 19.

7 Bradypus griseus griseus. Section through an adrenal gland, showing cortex and medulla. Chromaffin stain. Hematoxylin and eosin. × 14.

8 Bradypus griseus griseus. Photograph showing the shape of the spleen in the tridactyl sloth. × 1.4.

9 Bradypus griseus griseus. Lungs and trachea of a juvenile tridactyl sloth, showing the course of the trachea. There are no fissures to indicate the lobes of the lungs. × 0.9.

10 Bradypus griseus griseus. Section through the vascular bundle of the arm, to show the brachial artery, surrounded by a set of smaller arteries and a set of veins, all inclosed in a sheath to constitute a typical vascular bundle as it is encountered in the sloth. Mallory's connective-tissue stain. × 19.
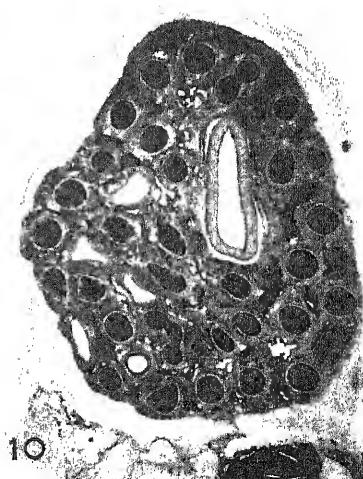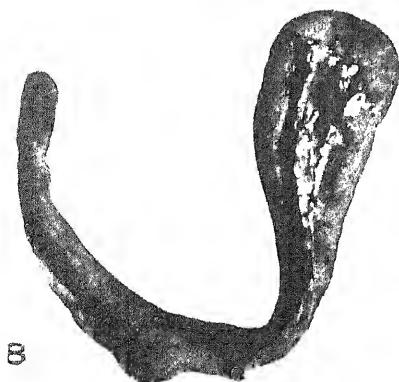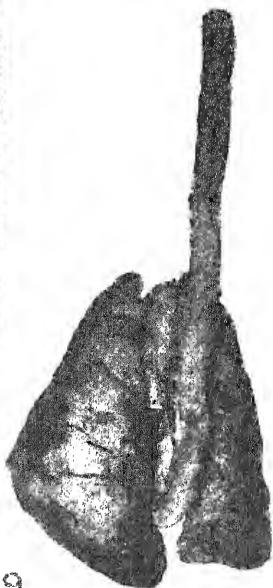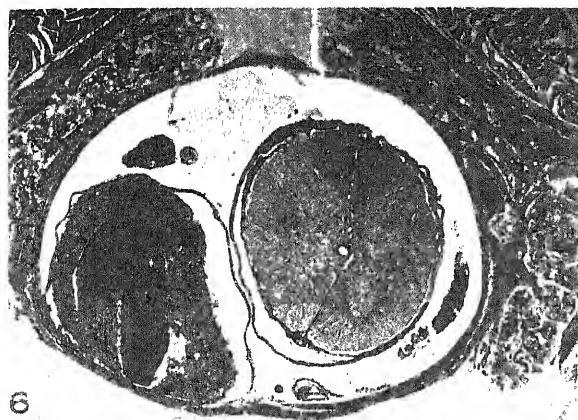
11 Photograph to illustrate the proportions and relative lengths of the limbs in Choloepus (left) and Bradypus (right).

12 Bradypus griseus griseus. Photograph to illustrate the sexual differences in the pelvis in an adult male (right) and an adult female (left). Note the greater thickness of the symphysis in the male than in the female.

13 Choloepus hoffmanni. Liver of the didactyl sloth, illustrating the small size and simplicity of the gall bladder.

14 Choloepus hoffmanni. Spleen of the didactyl sloth. Note the white mass, the pancreas, in contact with it at the hilum.   × 1.2.

15 Bradypus griseus. Typically bilobed ovary of the sloth. The ovarial pouch has been drawn aside to expose the ovary. The larger pole contained a corpus luteum of pregnancy.   × 3.

Choloepus

Brachypus

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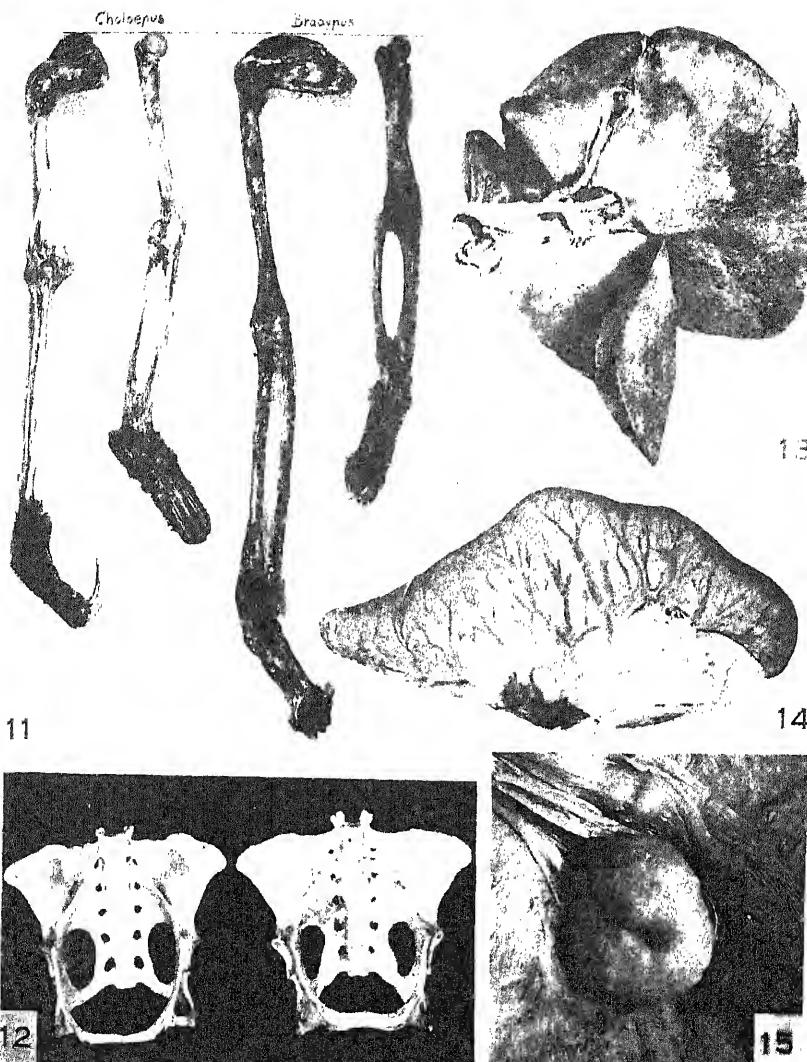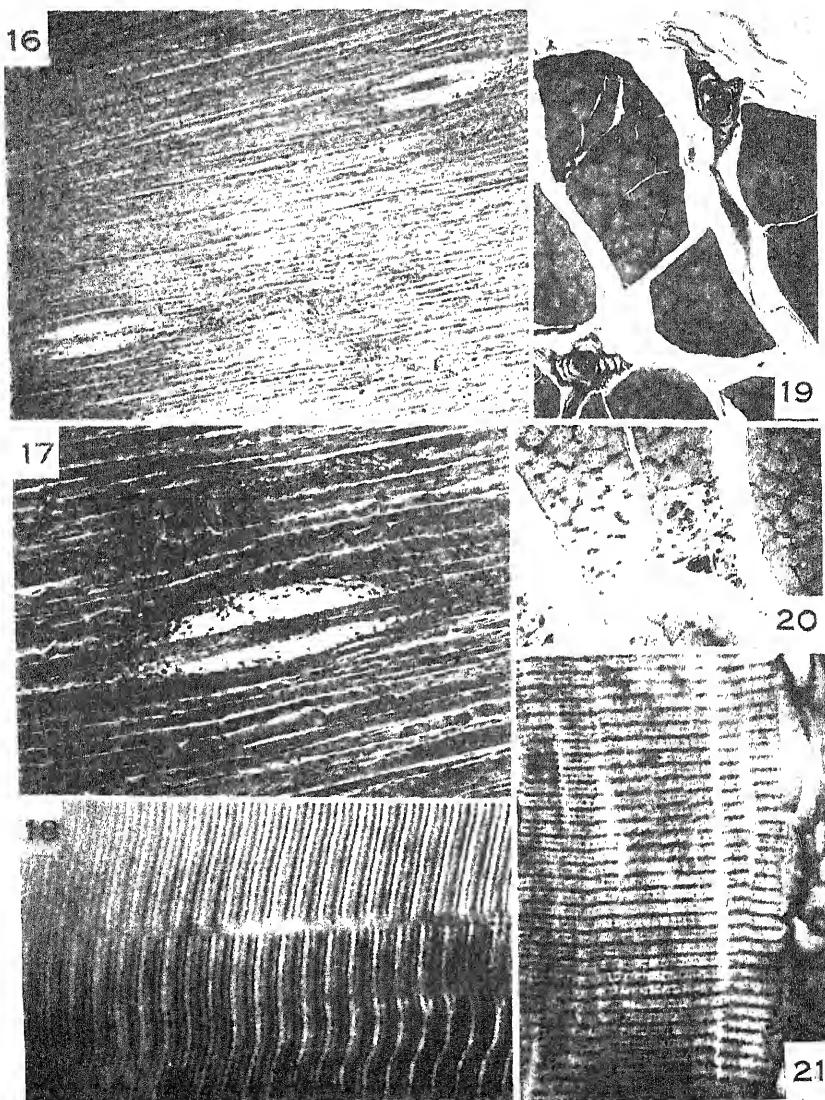
16 Bradypus griseus griseus. Section of the striated musculature, showing two neuromuscular spindles cut longitudinally. × 58.

17 Bradypus griseus griseus, showing a longitudinal section of a neuro-muscular spindle. Note the intrafusal fibers traversing the spindle, as well as the darker nuclear mass near its center. × 126.

18 Bradypus griseus griseus. Striations of the voluntary musculature. Bielschowski's method. × 1450.

19 Bradypus griseus griseus. Neuromuscular spindles cut transversely. × 126.

20 Bradypus griseus griseus. Neuromuscular spindle cut transversely. × 126.

21 Bradypus griseus griseus. Striations of the voluntary musculature. Mallory's connective-tissue stain. × 1450.

22 Bradypus griseus griseus. Section through the skin of chest, showing the dense fibrous, acellular character of the thick corium and the absence of fat. One of the infrequent sweat glands is shown (a). Hematoxylin and eosin. $\times$ 126.

23 Choloepus hoffmanni. Section through liver and wall of gall bladder. In the wall of the gall bladder of the didactyl sloth, there is scarcely any musculature. A slender strip of smooth muscle can be seen at a. Mallory's connective-tissue stain. $\times$ 53.

24 Choloepus hoffmanni. Section through skin of snout, showing the large sweat glands in that region. The cellular sheaths in the corium accompanying the blood vessels can also be seen. Hematoxylin and eosin. $\times$ 126.

25 Choloepus hoffmanni. Section through the anal gland, showing its sebaceous character. Hematoxylin and eosin. $\times$ 11.
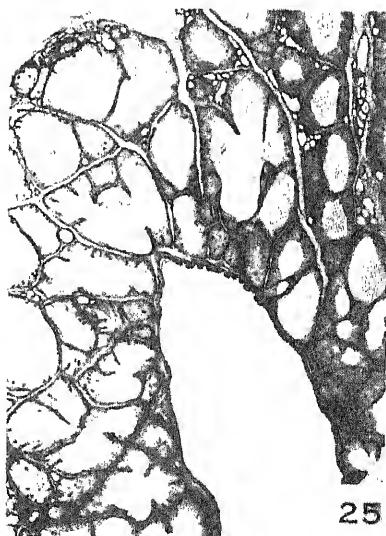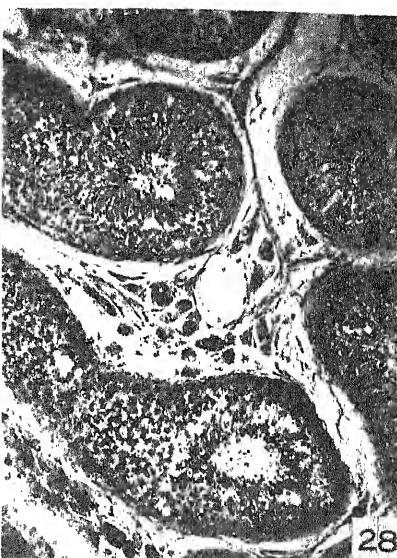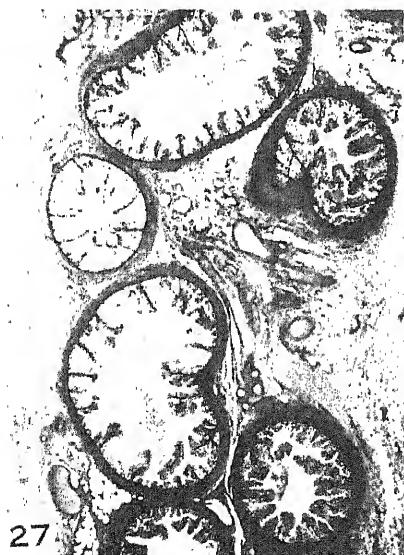
26 Bradypus griseus griseus. Section showing the ovary, containing a corpus luteum of pregnancy. Hematoxylin and eosin. × 19.

27 Bradypus griseus griseus. Section showing a seminal vesicle. Hematoxylin and eosin. × 14.

28 Choloepus hoffmanni. Section showing the testis with large pigmented cells in the interstitial tissue. Hematoxylin and eosin. × 126.

29 Bradypus griseus griseus. Section through the urethra and prostate gland. Hematoxylin and eosin. × 11.

30 Bradypus griseus griseus. Section through thyroid gland, showing the lack of encapsulation and the interspersal of fat through the gland. Mallory's connective-tissue stain. × 19.

31 Bradypus griseus griseus. Section through the hypophysis, showing the cystic structures at the junction of the epithelial and neural portions of the gland. Hematoxylin and eosin. × 53.

32 Bradypus griseus griseus. A typical group of follicles from the thyroid, showing the abundance of the chromophobe colloid droplets in the periphery of the colloid. Mallory's connective-tissue stain. × 110.

33 Bradypus griseus griseus. Section showing the well-developed thymus in a juvenile specimen. The black masses near the bottom of the figure are Hassall's corpuscles. Mallory's connective-tissue stain. × 19.
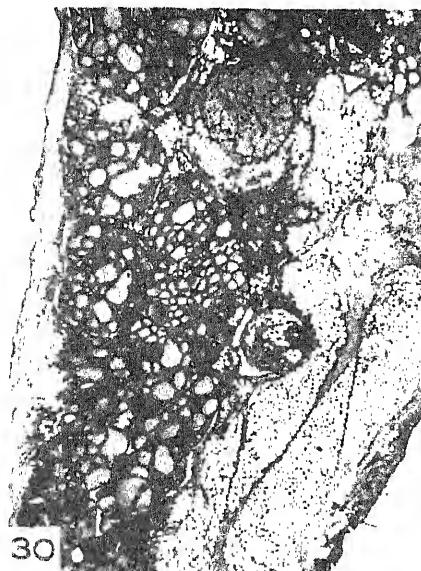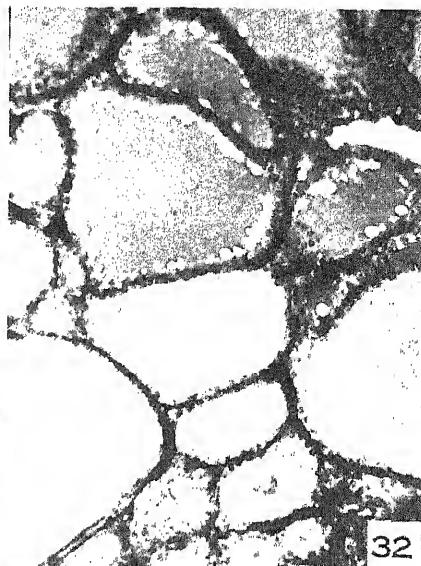
THE SEPTOMAXILLARY OF THE AMPHIBIA ANURA AND OF THE REPTILIA. II

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ONE CHART AND NINE PLATES (TWENTY-SEVEN FIGURES)

AUTHOR'S ABSTRACT

The septomaxillary is described in certain anurans and in some of the reptiles in which it occurs. In urodeles and some anurans this bone arises by ossification of the nasal cartilages. Lack of embryological material has prevented the verification of this in the young stages of the reptiles. Adult reptiles have been examined, and in these the bone appears to be and has often been described as a 'membrane bone.' It is suggested, however, that the septomaxillary is originally a 'cartilage bone,' and that in the reptiles additional membrane bone layers form its main part and obscure its cartilaginous origin. The infolding of the bone in the anurans and reptiles until it lies in close contact with the nasal septum, and thereby loses contact with the external nasal structures, appears to be correlated with the loss of the external nasal muscles. This loss in its turn results from the adoption of terrestrial life and consequent changes in the respiratory mechanism.

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INTRODUCTION

The homology of the septomaxillary among the lower vertebrates is now an accepted fact, and the names 'cornet,' 'os intranasale,' and 'turbinal,' given by various authors in the past, have been rejected in favor of the name 'septo-maxillary,' originally used by W. K. Parker in his amphibian studies. In vol. 45, no. 2, of this Journal, I have shown that the septomaxillary among the urodeles is an ossification of the cartilage in the posterior wall of the fenestra rostrolateralis, which provides for the partial origin of the musculus dilatator naris proper and of the musculus dilatator naris accessorius, and which is usually closely related to the nasolacrimal duct. The development of the nasal muscles and the size of the septomaxillary increase in the higher members of the group.

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Curiosity about the fate of the external nasal musculature of the urodeles prompted my further investigation of the septomaxillary and of the nasal region generally in the anurans, gymnophionans, and reptiles. The gap separating the anurans from the urodeles is large, owing to the extremely specialized condition of the anuran nasal capsule with its three chambers. The external nasal muscles of the anurans are modified from the urodelean forms and their relation to the septomaxillary is changed.

As we pass higher in the vertebrate line to the reptiles, the septomaxillary, present in the Rhynchocephalia (Sphenodon), in lizards, snakes, phytosaurs, and in some theromorphs, tends more and more to lose its former characteristic of being outside the nasal capsule; it undergoes a process of infolding until it comes to lie in the floor of the nasal sac in close contact with the nasal septum; it also loses to a certain extent its intimate relation with the nasolacrimal duct, which opens, together with Jacobson's organ, into the mouth. Hence the bone is difficult to see in this group unless the skull is dissociated.

I have tried to take representative species of each group as far as the available material allowed. The different genera of the groups Lacertilia and Ophidia approximated to two main types of nasal structure which have been described by earlier investigators (Born, 3, 4, 5; Parker, 26, 27). In these groups, therefore, I have restricted myself to a consideration mainly of the function and position of the septomaxillary.

MATERIALS AND METHODS

The following species have been examined:

Class Amphibia:
  1. Stegocephalia,   None.
  2. Urodela,   See Introduction.
  3. Gymnophiona,   Ichthyophis glutinosus, page 409.
                             Hypogeophis rostratus, page 410.

4. Anura:

    Aglossa,   Xenopus calcaratus, page 407.

    Phaneroglossa,   Bufo marinus, page 406.

                             Rana temporaria, page 403.

                             Rana palustris, page 404.

                             Hyla wilderi, page 406.

Class Reptilia:

  1. Rhynchocephalia,   Sphenodon punctatus, page 412.

  2. Lepidosauria:

    Lacertilia: Page 412.

      Geckonidae,   Gonatodes vittatus.

      Amphisbaenidae,   Amphisbaena fuliginosa.

      Lacertidae,   Lacerta muralis.

    Ophidia: Page 412.

      Typhlopidae,   Typhlops porrectus.

      Glauconiidae,   Glauconia nigricans.

      Boidae,   Eunectes murinus.

      Uropeltidae,   Plectrurus perroteti.

      Colubridae,   Lycodon aulicus.

                            Atractis trilineatus.

Excepting Rana, the preparation of the skull by Noble's modification of the Schultze KOH method (the staining of the bone with alcoholic alizarin, before clearing in glycerin), which was used so successfully for the urodeles, has not been practicable, either through lack of sufficient material or because the position of the septomaxillary inside the nasal cavity made the observation of it in the intact skull a difficult matter. Most of the heads examined have been cut into sections 10 μ or 15 μ thick, and the bone reconstructed from the sections by the glass-plate method: consecutive sections of the object are drawn on thin glass plates with a ground surface (the necessary magnification being calculated from the thickness of the glass plates and the thickness of the sections); these are piled up with cedarwood oil between each to clear them. A solid reconstruction of the object is thus visible when the whole pile of plates is lit, preferably from

behind or below. Plentiful fresh material of the frog (Rana temporaria) and of the wall lizard (Lacerta muralis) made it possible to dissociate the skulls with KOH and to study the septomaxillary without using this rather tedious reconstruction method, which is nevertheless invaluable where material is scarce.

The fresh material for sectioning was fixed with Bouin's fluid and decalcified in a saturated solution of picric acid with 1 per cent concentrated nitric acid. The reptilian material presented difficulties in decalcification and in the softening of the horny scales. The use of 1 per cent HNO_3 in 70 per cent alcohol as a decalcifying fluid was sometimes sufficient, and the scales became soft enough to section in the ordinary way. This method failed twice for Typhlops porrectus, of which material was plentiful; in the third attempt I used the double-embedding method, impregnating celloidin with paraffin wax, which proved quite successful (Eltringham, "Butterfly vision," Journ. Ent., 1919). I also experimented, but unsuccessfully, with CS_2 for softening the scales, and in one or two instances, e.g., Eunectes murinus, resorted to a careful skinning of the head in order to get rid of the scales entirely.

The most successful stain for sections was found to be alcoholic borax carmine followed by picro-nigrosin, which gives very good differentiation of the tissues. Magenta-indigo-picrocarmine was also used quite successfully, though differentiation is not so good as by the former method.

I have used mainly the nomenclature of Ecker and Wiedersheim and of W. K. Parker.

In my search for material, I am indebted to Prof. J. S. Dunkerly for Bufo, Amphisbaena, and Hypogeophis; to Doctor Carpenter for the use of the reserve collection of snakes in the Manchester Museum; to Prof. Graham Kerr, of Glasgow, for two specimens of Xenopus; to Dr. Joseph Pearson, of Colombo, Ceylon, who kindly sent me a specimen of Ichthyophis; and to Prof. Julian Huxley for allowing me to

examine, at King's College, London, the material of Sphenodon prepared by Dendy and others.

Most of the material has been preserved for museum exhibition, and not for histological study, so that shrinkage of the soft tissues, due to bad fixation, is seen in some instances; but this has not interfered appreciably with the purpose of the work.

DESCRIPTION

The septomaxillary in the anurans

The assumption of land habits by the anurans and the consequent adoption of an air-breathing mode of life have reacted upon their respiratory mechanism, causing changes in the musculature of the external nares. In this group the external nares are controlled by the striated musculus submentalis and the masticatory muscles, instead of by the more slowly responding smooth muscles, which have been shown to have an intimate relation to the septomaxillary of the urodeles.

Rana. The closing of the nostril and the events leading up to it have been well described for Rana fusca by Gaupp(10), who points out the importance of the connection of the cartilago alaris with the premaxilla. The closing of the mouth is brought about by the raising of the lower jaw by means of the masseter muscle; as this takes place, the tuberculum praelinguale of the lower jaw is pressed into the fossa subrostralis media of the upper jaw, at the bottom of which lies the suture between the two premaxillae. The consequent slight separation of the premaxillae, into whose hollow facial process the processus praenasalis superior of the cartilago alaris projects, causes an outward movement of this portion of the cartilago alaris; as this part is forced outward, the larger posterior part of the cartilage swings inward and thus effects the closing of the nostril (fig. 2).

Describing the position of the septomaxillary in Rana fusca and its relation to the other nasal structures, Bruner says:

In the roof of the cavum medium, supported by the lamina superior, the planum terminale and the lateral wall of the nasal capsule, lies a small horseshoe-shaped bone, the intranasale of Gaupp, whose two caudo-medially directed arms are separated by a narrow cleft. The medial arm of the bone lies in close contact with the free margin of the lamina superior; its broader lateral arm extends towards the anterior incurved angle of the planum terminale, embraces with its excavated caudal margin the anterior convex surface of this cartilage, and then terminates at the medial margin of the same, that is to say, at the anterior incurved end of the turbinal prominence. Beneath the lateral arm of the intranasale the ductus naso-lacrymalis passes to its opening at the anterior end of the planum terminale.

The slit-like opening between the two arms of the intranasale leads from the cavum medium into a lateral vestibule of the cavum superius, which is bounded in front and laterally by the cartilago alaris, behind by the thickening of the lateral nasal wall which contains the glandula nasalis externa. This vestibule, which was briefly described by Born, is separated from the cavum superius itself by folds only, of which one connected with the caudal wall of the vestibule attains a considerable size. This fold, which I shall call plica obliqua, extends from below obliquely upward and terminates at the medial margin of the nasal opening, where it is attached to the cartilaginous roof of the nasal capsule(7, pp. 388 and 389).

I have verified this for Rana temporaria (figs. 1, 3, 4, and 5) and Rana palustris, which show a similar intimate connection between the septomaxillary and the adjacent nasal cartilages as described for the urodeles. Bruner has homologized the two nasal muscles of Rana with the musculus constrictor naris and the musculus dilatator naris of the urodeles, but Gaupp(10) regards this as a fallacy because Bruner's so-called constrictor muscle is apparently slightly dilatatory (v. infra). He therefore gives the names musculus lateralis narium to Bruner's musculus constrictor naris, and musculus medialis narium to Bruner's musculus dilatator naris. Bruner found the development of the external nasal muscles and of the external nasal gland of Rana to resemble the homologous parts in Triton.

The lateral nasal muscle of Rana temporaria is larger than the medial and lies behind the apertura naris externa, the

vestibulum, and the recessus sacciformis. Its composite fibers arise from the posterior part of the cartilago obliqua, the planum terminale, and the connective tissue between the skin and the glandula nasalis externa. It extends anteriorly outside the septomaxillary and the glandular tissue, to be inserted in a radiating manner into the connective tissue of the posterior border of the apertura naris externa and into the plica obliqua. The function of the lateral muscle is to hold open the apertura naris externa, the vestibulum, and the recessus sacciformis, and to give tension to the plica obliqua; it is thus rather more dilatatory than constrictory. It also partially controls the discharge of the secretions of the glandula nasalis externa.

The medial muscle lies to the inside of the same glandular tissue and part of its external surface is applied to the inside of the short lateral arm of the septomaxillary and more posteriorly to the medial side of the planum terminale, from whose base it mainly takes its origin; only a few of its fibers arise from the lateral arm of the septomaxillary. The muscle ascends anteriorly close to the medial arm of the septomaxillary and merges into the connective tissue, which itself ascends farther forward still into the plica obliqua and surrounds the cartilago alaris. Thus the insertion of this muscle is similar to that of the lateral muscle, although the actual muscle does not extend as far forward. The function of the medial muscle is to reinforce the tension already given to the plica obliqua by the lateral muscle. The importance of the tension of this fold is apparent when one considers the mechanism of the closing of the external nares as it is above described. When the cartilago alaris swings round, it causes the lateral wall of the vestibule to press against the plica obliqua. The tension of the soft borders of the external naris, brought about by the condition of these two smooth nasal muscles, changes little or not at all during the various phases of respiration.

Thus neither of these muscles is a true dilatator or constrictor of the external naris. Gaupp's nomenclature, there-

fore, describes them better, although Bruner's terms indicate their homology with the corresponding muscles in the urodeles. Bruner's view is further supported by embryological evidence. He and others suggest that changes in the respiratory mechanism of the anurans as a result of their adoption of a terrestrial life have caused the alteration in the function of these muscles.

The description for Rana temporaria and Rana palustris stands for Hyla wilderi, which has also been examined for possible variations.

Bufo marinus. In this species the nasal sac has three chambers similar to those found in Rana, namely, the cavum superius, the cavum medium, and the cavum inferius (Jacobson's organ). This homology of the cavum inferius and Jacobson's organ has been established by Wiedersheim, although Howes(17) believes that the structure in the Amphibia, sometimes called the organ of Jacobson, is a maxillary sinus, not homologous with Jacobson's organ of higher animals, and that Jacobson's organ exists in its most nearly original form in the Lacertilia.

The cavum superius is the main cavity. It extends from the region in front of the external naris backward to open into the cavum inferius. Neither the cavum medium nor the cavum inferius extends quite so far forward. They both appear in sections passing through the anterior border of the naris. The cavum medium extends backward as far as a point slightly behind the posterior border of the external naris, where it opens into the lowermost sac, the cavum inferius; it also projects backward a little way in a ventral groove of the septomaxillary. The cavum inferius, receiving thus the other two cavities, itself opens through the choana into the mouth.

The septomaxillary of Bufo is larger than that of Rana and its shape is different. Its blunt anterior end lies in the connective tissue below the cartilago alaris. Anteriorly and laterally, it roofs in the cavum medium, supplementing the lamina superior of the crista intermedia. More posteriorly,

the bone is seen to divide (compare the condition in Rana) to allow the cavum medium to open into the infundibulum of the cavum superius. Behind this point the medial shank of the bone, closely applied to the lamina superior, is much shorter than it is in Rana. The lateral shank, however, is much larger than it is in the corresponding bone in Rana. It curves round the posterior border of the apertura nasalis externa and sends out a bony support which apparently replaces a certain amount of muscle and connective tissue in the plica obliqua. This lateral shank bears posteriorly three depressions: two caused by the external nasal gland, and a third, more posterior depression, into which fits a cartilaginous process of the planum terminale near its junction with the cartilago obliqua (figs. 6 and 7).

The lateral nasal muscle is smaller than in Rana and its fibers are somewhat diffuse. It takes its origin from the maxilla and from the adjacent nasal cartilages. Radiating upward anteriorly, over the outer surface of the septomaxillary, it is inserted into the connective tissue round the posterior border of the external naris, giving tension to this region.

The medial muscle is represented by the few fibers arising from the inner and outer surfaces of the process of the septomaxillary which lies in the plica obliqua. It is inserted into the dorsal surface of the cartilago obliqua (fig. 7). Its function of retaining tension in this region is supplemented and almost entirely taken over by the process of the septomaxillary which projects into the plica obliqua.

The nasolacrimal duct runs forward in a groove on the posterior lateral border of the septomaxillary and then passes underneath the bone to open into the nasal sac in the hinder end of the cavum medium.

Xenopus calcaratus. Owing to the curious infoldings of its nasal cartilages, Xenopus has an extremely specialized nasal sac. Parker(25) first described the cartilaginous skull and also pointed out that in the structure of its nose Xenopus differs from Pipa as much as they both do from the other Batrachia. He speaks of the cornua trabeculae being—

curiously overlapped by the most highly developed form of nasal valve with which I am acquainted. . . . [They] are extremely unlike those of the frog and toad; they are continuous with the great labial pouches in front, and with the septum nasi in the middle. These cornua are not seen above, being covered by the labial pouch; but below they are seen . . . . to be tilted plates, with their convex face in front; they grow outwards and turn backwards, and help to give complexity to the infoldings of the Schneiderian membrane (figs. 11 and 12).

The choana of Xenopus is placed fairly far back in the roof of the mouth, the posterior sections through the choana cutting the eyes. The cavum nasale, whose walls are thickened with sensory epithelium, is a pear-shaped sac, rounded medially and opening ventrolaterally into the choana. More anteriorly, it opens into the lateral nasal canal, a narrow flattened extension of it which enlarges anteriorly and buds off laterally a small sac which ends blindly in front at a level which marks the posterior blind ends of Jacobson's organ and the cavum medium. Jacobson's organ is a medial ventral offshoot of the lateral nasal canal. It is embedded in Jacobson's gland, which lies in its usual position underneath the cavum nasale and the lateral nasal canal, and ends blindly in front, giving off a small medial pocket just behind the level of the posterior border of the external naris. The cavum medium also opens into the lateral nasal canal just behind the external naris. It is an irregular structure, lined with sensory epithelium, and inclosed by the nasal cartilages, that is to say, by the cornu trabeculae medially and laterally by the lateral wall of the large labial pouch. The nasolacrimal duct lies along the top of it, passing first through a notch in the posterior border of the septomaxillary, and then under this bone and dorsal to the cavum medium, with which it opens anteriorly into the lateral nasal canal (figs. 8 and 9).

The septomaxillary roofs in this region, between the cornu trabeculae and the lateral wall of the labial pouch (figs. 11 and 12). It is a simple, slightly curved plate broadening posteriorly. Anteriorly, it forks round the posterior border

of the external naris. This part of the bone is embedded in
the constrictor or lateral muscle which lies in the connective
tissue below the skin, gives tension to the posterior border
of the naris, and thus has a minor function in the mechanism
of inspiration as described for Rana. Of the medial muscle
there seems to be no trace, but, posteriorly, a few muscle
fibers, arising from the outer surface and from the dorsal
surface of the septomaxillary, pass over the outer surface
of the cartilago obliqua and are inserted into the lateral
border of the nasal bone; none of these, however, runs as
far forward as the external naris.

The septomaxillary in the Gymnophiona

Ichthyophis glutinosus. The septomaxillary of Ichthyophis
glutinosus was first described by Huxley(19) as a bone which
seems to be an ossification of the cartilaginous ala nasi, and
later by P. and F. Sarasin(31), who called it a 'turbinal.'
It lies in the lateral border of the anterior portion of the
nasal sac (compare urodeles), forming the nasal wall; figure
13 (after the Sarasins) shows its general shape. The 'obere
lamelle,' the most medial wing, forms an acute angle with
the rest of the bone and is intimately applied to the dorsal
nasal process (Winslow, 34). The 'acussere lamelle' and the
'untere lamelle' wall in the skull between the nasal and the
premaxilla, and, more posteriorly, between the nasal and the
maxilla. The bone lies in command of the fenestra narina.
Some of the fibers of the external nasal muscle are inserted
in the anterior inner border of its upper wing (fig. 14), some
in the dorsal nasal process and some in the ventral nasal
process just behind the external naris. This muscle is homolo-
gous with the constrictor (lateral) muscle of the urodeles
and anurans, but it may have fibers derived from those of
the dilatator (medial) muscle (compare position of the septo-
maxillary and the medial muscle in Bufo marinus). A series
of developmental stages would help to make this point clearer.
It plays a part in the opening and closing of the external
naris during respiration, but there is probably also a mechan-

ism in addition similar to that of the anurans, although the processes of respiration of this animal have not, to my knowledge, been worked out. Because of the anterior and external position of the bone, it in no way comes into contact with the organ of Jacobson.

Hypogeophis rostratus has also been examined. In this animal the septomaxillary is absent; the nasal and premaxilla are larger and meet each other, walling in the side of the skull. There is an external nasal muscle round the posterior border of the external naris similar to that described for Ichthyophis.

The septomaxillary in the reptiles

For the last fifty years, authors of works on the reptilian head (Born, Gaupp, Gunther, Howes and Swinnerton, Osawa, W. K. Parker, Schauinsland, and others) have either only mentioned this bone or have given no more than a brief description of it. No comparative anatomist has, to my knowledge, gathered up the facts and considered the bone in the reptilian group as a whole, nor has its relation to the amphibian septomaxillary been discussed. To do this has been my purpose in examining the available specimens (see list of material in Introduction) by sectioning and maceration. Among the reptiles, the bone is found in Sphenodon, in lizards, in snakes, in phytosaurs, and in some theromorphs. (Kingsley(22) omits the snakes in his list of reptiles possessing a septomaxillary, though many authors have described it in this group.)

The most variable feature of the nasal structure of the reptiles is the position of the choanae. They may be far forward, with the anterior tips of the vomers lying between them, or the choanae of the two sides may approximate. On the other hand, this condition may be modified by a medial extension of the palatal processes of the premaxillae to form a hard palate. The respiratory ducts then pass along the roof of the mouth and above the hard palate, and the actual choanae are displaced backward to a position behind the

posterior edge. If the palatines and pterygoids also meet in the middle line in a similar way and cover the vomers and sphenethmoids, the choanae are pushed still farther back. In the modern Crocodilia they have been thus displaced backward to a position near the posterior end of the whole cranium.

With regard to the nasal muscles of reptiles, Hoffmann(16) discovered the smooth nasal muscles of the crocodiles without giving details of their number and arrangement. Bruner (6) reports that, as far as the smooth external nasal muscles are concerned, the Crocodilia may be homologized with the Salamandridae, because both groups possess a semicircular musculus constrictor naris, which is attached in both median and lateral directions to the margin of the apertura naris cranialis, and also a straight musculus dilatator naris, which arises from the posterolateral margin of the apertura and is inserted into the posterior border of the nasal opening.

Among the lizards there exists in the wall of the nasal vestibule a tissue composed of smooth-muscle fibers and blood sinuses in connective tissue, which may, by increase of muscular tension and pressure of the blood, produce a thickening of the margin of the naris and thus bring about its closure.

Among the snakes this cavernous tissue is present in the region of the external naris, but there is also a smooth musculus subnasalis which arises from the anterior ventrolateral border of the septomaxillary and is inserted into the ventral part of the plica nasalis (figs. 26 and 28). This muscle corresponds in form to the musculus medialis (dilatator) narium of the urodeles and anurans.

The septomaxillary of all the reptiles investigated forms part of the floor of the nasal cavity. Usually it flanks the septum nasi medially and protects Jacobson's organ to a greater or less extent posteriorly; laterally, it may or may not form the side wall of the nose.

Rhynchocephalia (Sphenodon punctatus)

The septomaxillary of Sphenodon punctatus was shown by the figures of Fuchs(9) to have an almost urodelan relation to the other structures in the anterior region of the nose. It lies in the neighborhood of the outer vestibule. Fuchs described the bone as having an intimate relation with the nasal muscle, but I was unable to perceive this muscle in any of the series of sections of Sphenodon that I examined at King's College, because, perhaps, the fixation of this material was often poor and the stain faded. The bone is a small curved structure supporting the bottom of the nasal cavity. It does not roof in Jacobson's organ, this function being taken over by cartilage on which the septomaxillary lies (fig. 16). Jacobson's organ opens medially, and the nasolacrimal duct laterally, into the anterior part of the choana.

Lacertilia and Ophidia

The septomaxillary of the lizards is a flat plate (figs. 17 to 20) with curving sides. It flanks the septum nasi with its medial border, roofs over Jacobson's organ, and protects the side walls of the nasal sac; this lateral border may have a dorsal projection directed backward.

The septomaxillary of the snakes seen from below appears as a wedge, the narrow part of which is anterior. The medial edge is separated from the nasal septum by connective tissue only. The upper surface of the bone is hollowed to form the floor of the nasal sac. Posteriorly, in its broadest part, it forms another hollow, which functions as a lid to the 'vomerine dish' (figs. 21 to 27).

The nasal capsules of the lizards and snakes are very similar, although those of the lizards are more labyrinthic. Their septomaxillaries differ chiefly in the shape of their posterior portions. In the snakes this part forms a much more extensive, curving roof to Jacobson's organ than it does in the lizards, where it is little more than a flat plate flooring the upper nasal cavity.

SUMMARY

The septomaxillary of the urodeles is a bone which arises by ossification of the cartilage. In the adults of the less specialized members of this group there is an intimate connection between the bone and the nasal cartilages. In the more specialized urodeles it acquires added layers of 'skin-formed' or membrane bone. Among the anurans studied a similar intimate relation between the septomaxillary and the nasal cartilages has been found. In certain places, e.g., where the septomaxillary is in contact with the lamina superior of the crista intermedia, these cartilages seem to be continuous with the septomaxillary—a fact which further supports the view that the septomaxillary arises in cartilage. Among the reptiles this continuity is not found, and the septomaxillary is apparently of wholly membranous origin, although embryological investigations may show that there is a basis of cartilage bone.

Among the urodeles the septomaxillary is either a minute nodule or a small curved piece of bone in the posterior border of the fenestra rostrolateralis, and has intimately connected with the nasal muscles and the nasolacrimal duct. Among the anurans the septomaxillary becomes slightly folded into the nasal capsule to roof in the cavum medium, and the external nasal muscles have a lesser function. It is in Rana that we find these muscles to resemble most closely the urodelan condition; Rana may therefore be regarded as the most primitive anuran in this respect. Bruner(7) records traces of the nasal muscles in Alytes. The degeneration in these forms appears to be correlated with, and accompanied by, the development of the septomaxillary as a bony support for the plica obliqua. Bufo marinus shows the extreme development of the septomaxillary along one line of evolution: the development of the medial (dilatator) muscle, whose function is to give tension to the plica obliqua, is determined by the form of the septomaxillary which, in Bufo, renders the muscle superfluous. The development of the lateral (constrictor) muscle is also influenced in this way.

The nasal region of Xenopus is more primitive than that of Rana. Its septomaxillary is a small plate, and there is a constrictor muscle, but I could not find any trace of any other muscle.

The septomaxillary of Ichthyophis glutinosus forms the side wall to the anterior portion of the nasal sac, but it is small and does not extend far back. In these features it closely resembles the septomaxillary of the higher urodeles, although the external nasal muscles are not so well differentiated.

Among the reptiles the septomaxillary of Sphenodon punctatus shows a movement inward, it floors the upper nasal cavity, but has no close contact with the nasal septum. It also lies above the cartilage which covers Jacobson's organ. I was unable to find any trace of the external nasal muscles.

Bruner(6) has described constrictor and dilatator muscles for the crocodiles homologous with those of the Salamandridae. The aquatic mode of life of both these groups and their common need for quick closure of the external nares when the head is immersed probably account for the similarity of their nasal musculature, but the absence of the septomaxillary in the crocodiles must change the origin of the musculus dilatator naris.

Among the lizards and snakes the septomaxillary roofs in Jacobson's organ replacing the cartilage, and its medial border is closely apposed to the nasal septum. In the region of the external nares in these two groups there is a cavernous tissue, composed of smooth-muscle fibers and blood sinuses, and the increase of muscular tension and blood pressure in this may produce a thickening of the margin and a partial closure of the nares. The snakes have, in addition, a smooth musculus subnasalis which is homologous with the musculus medialis (dilatator) narium of the urodeles and anurans. The following table gives the varying forms of the septomaxillary in relation to the nasal sac in the Amphibia and reptiles (the septomaxillary is drawn in black; the nasal cartilages are drawn in dots).

AMPHIBIA.
GYMNOPHIONA. (Ichthyophis glutinosus)
URODELA.
ANURA. (XENOPUS)
ANURA (RANA)

REPTILES.
RHYNCHOCEPHALIA.
(Sphenodon punctatus)
LACERTILIA.
OPHIDIA.
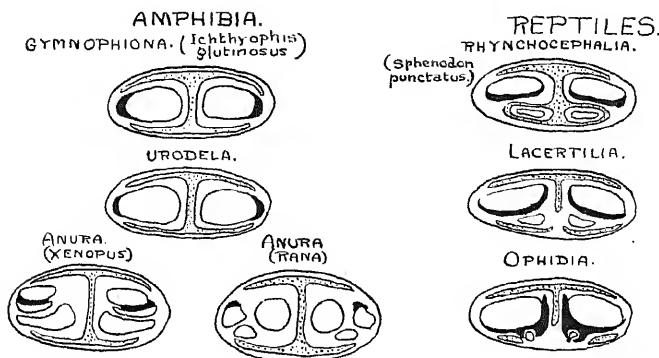
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ABBREVIATIONS

a.n.e., apertura naris externa (external naris). (*p.w.*), posterior wall

c., choana

c.a., cartilago alaris

c.i., cavum inferius

c.m., cavum medium

c.n., cavum nasale

c.o., cartilago obliqua

c.s., cavum superius

d.nl., ductus nasolacrymalis (nasolacrimal duct)

f.rl., fenestra rostrolateralis

f.s.m., fossa subrostralis media

gl.n.l., glandula nasalis lateralis (= externa)

J.g., Jacobson's gland

J.o., Jacobson's organ

l.i., lamina inferior cristae intermediae

l.n.c., lateral nasal canal

l.r., lateral arm of the septomaxillary

l.s., lamina superior cristae intermediae

m.m.n., musculus medialis narium

m.l.n., musculus lateralis narium

m.r., medial arm of the septomaxillary

m.s., musculus subnasalis

mx., maxilla

n., nasal

pmx., premaxilla. (*f.*), facial process

p.n., plica nasalis

p.o., plica obliqua

p.p.s., processus praenasalis superior of the cartilago alaris

p.t., planum terminale

smx., septomaxillary

s.n., septum nasi.

PLATE 1

EXPLANATION OF FIGURES

1 Dorsal view of the right septomaxillary of Rana temporaria. $\times$ 50.

2 Diagram to illustrate the mechanism for closing the external nares in Rana.

3 Transverse section through the right nasal region of Rana temporaria at the posterior border of the external naris. $\times$ 20.

4 Transverse section posterior to that shown in figure 3; the nasolacrimal duct (*d.nl.*) passes through a crevice in the septomaxillary (*smx.*) to open into the back of the cavum medium. $\times$ 20.
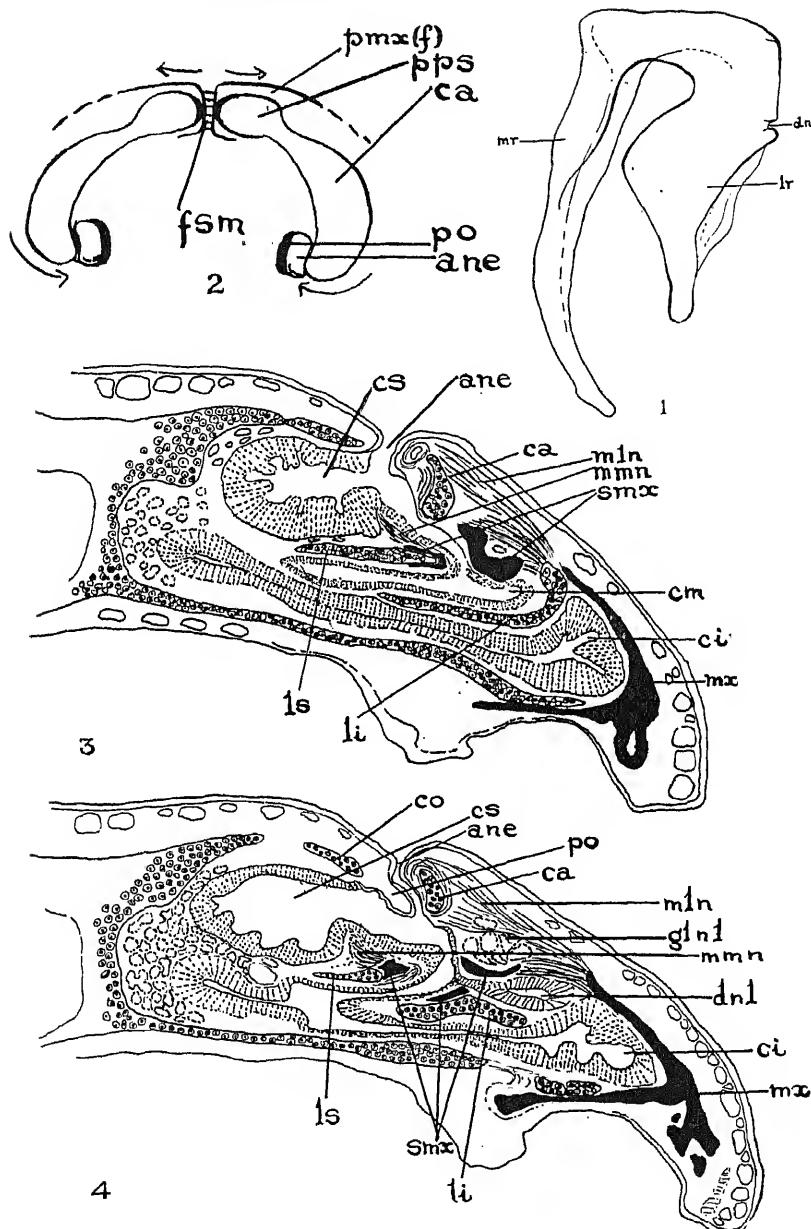
5 Transverse section posterior to that shown in figure 4, behind the naris. × 20.

6 Posterior view of the right septomaxillary of Bufo marinus, from a reconstruction. × 20.

7 Transverse section through the right nasal region of Bufo marinus at the posterior border of the external naris, showing the septomaxillary projecting into the plica obliqua. × 20.

5

6

7
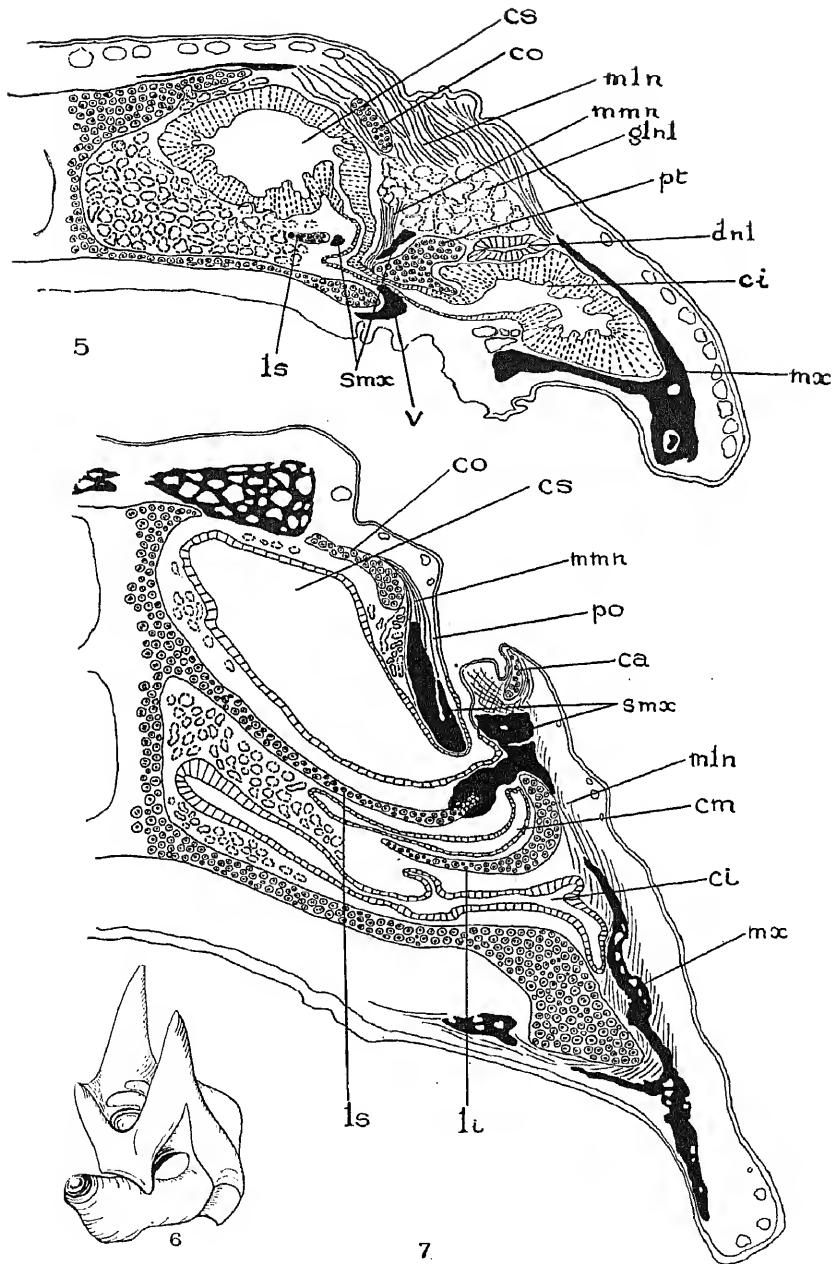
PLATE 3

EXPLANATION OF FIGURES

8 Diagram of right nasal sac of Xenopus calearatus from reconstruction. Ventral view.

9 Dorsal view of the same. The approximate position of the septomaxillary is drawn in dots.

10 Right septomaxillary of X. calearatus, posterior view. $\times$ 20.

11 Transverse section through nasal region of X. calearatus, cutting through C and D (figs. 9 and 8). $\times$ 20.
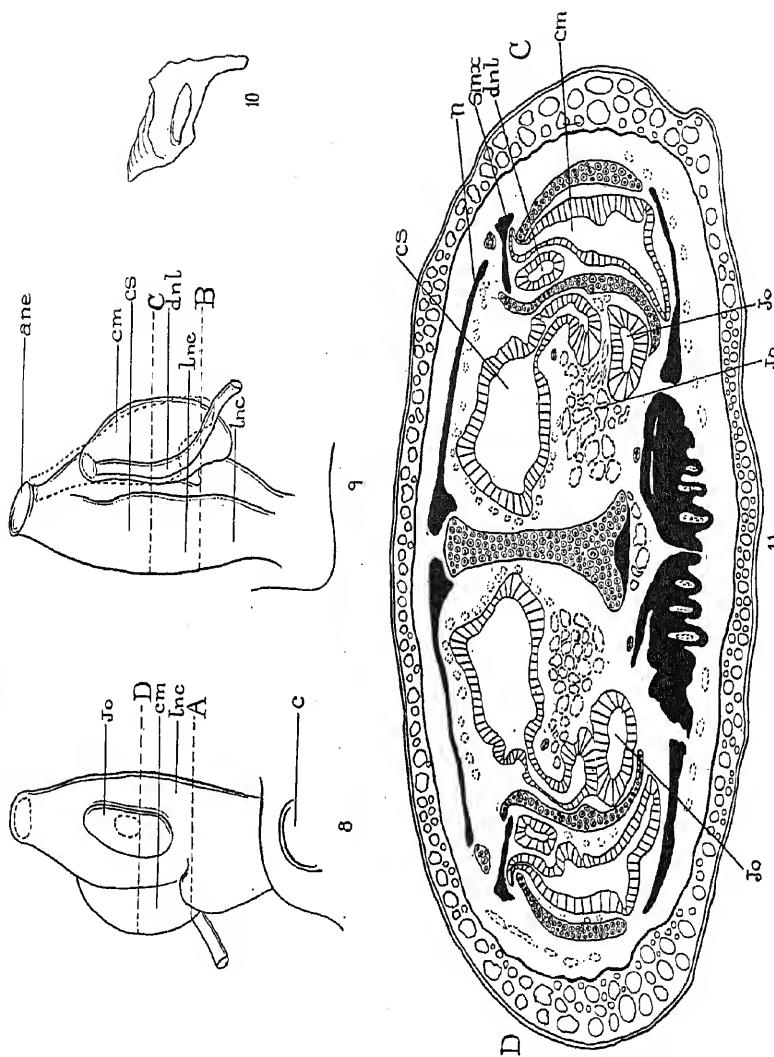
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PLATE 3

SEPTOMAXILLARY OF THE ANURA AND REPTILIA. II
ENID OLDHAM LAPAGE
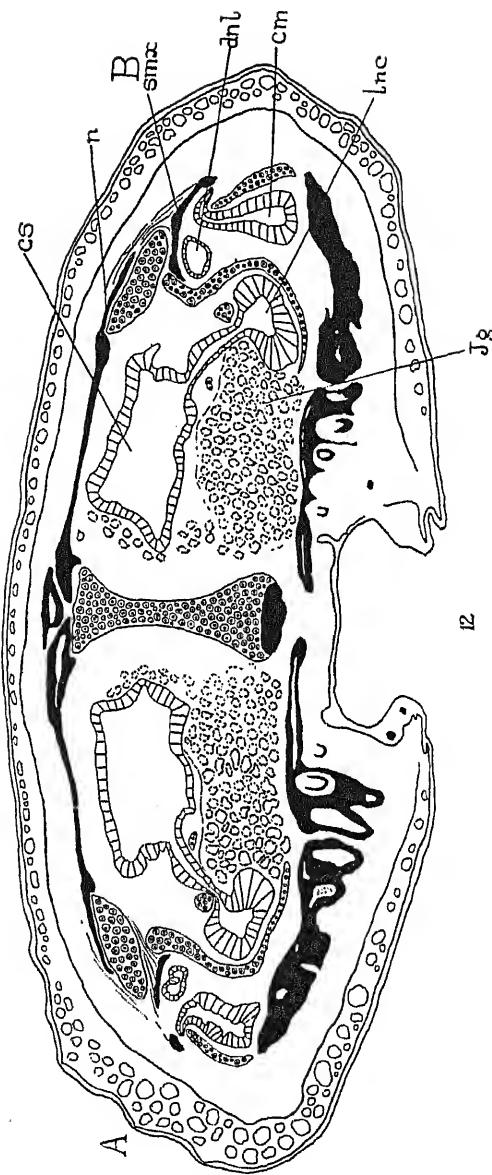
12   Transverse section a little posterior to that shown in figure 11, cutting through *A* and *B* (figs. 8 and 10). × 20.
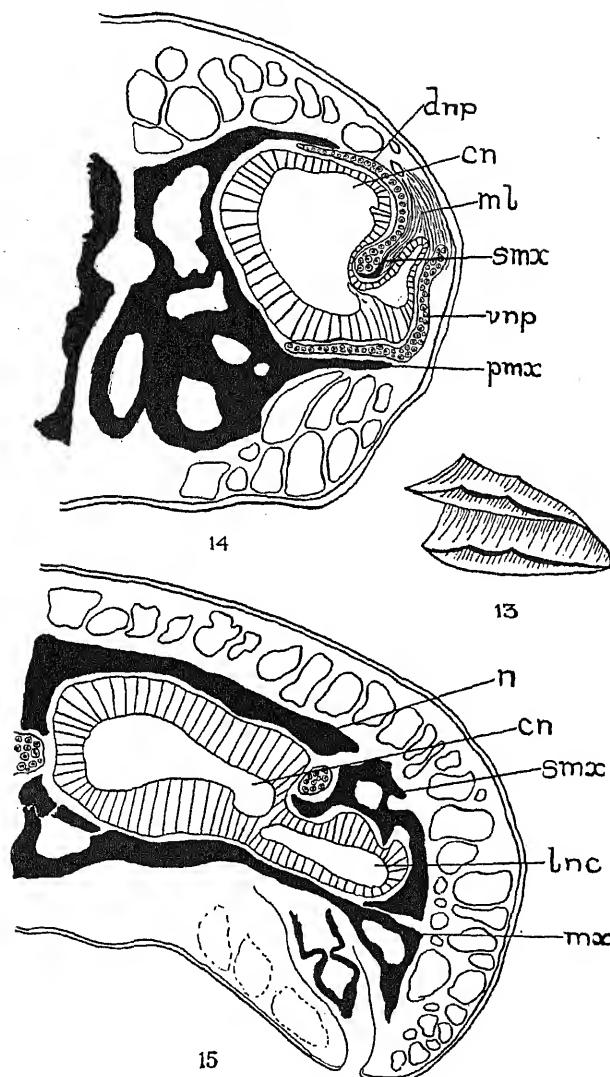
13   Left septomaxillary of Ichthyophis glutinosus from the inner side (after P. and F. Sarasin).

14   Transverse section through the right anterior nasal region of I. glutinosus; the section passes just behind the naris.   × 30.

15   Transverse section posterior to that shown in figure 14.   × 30.
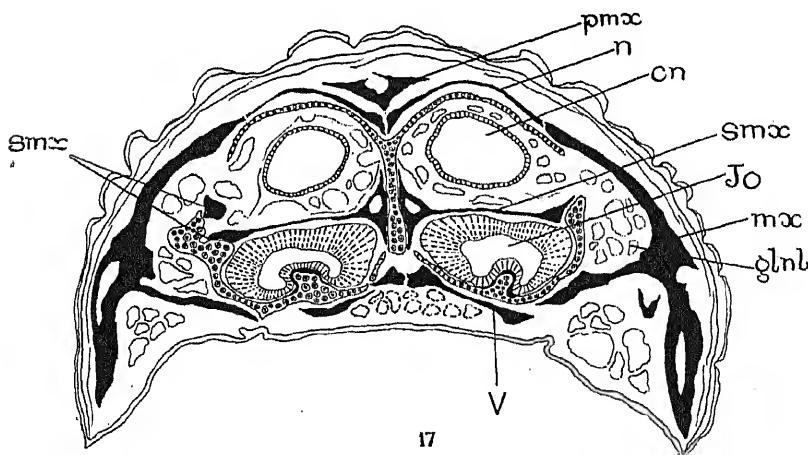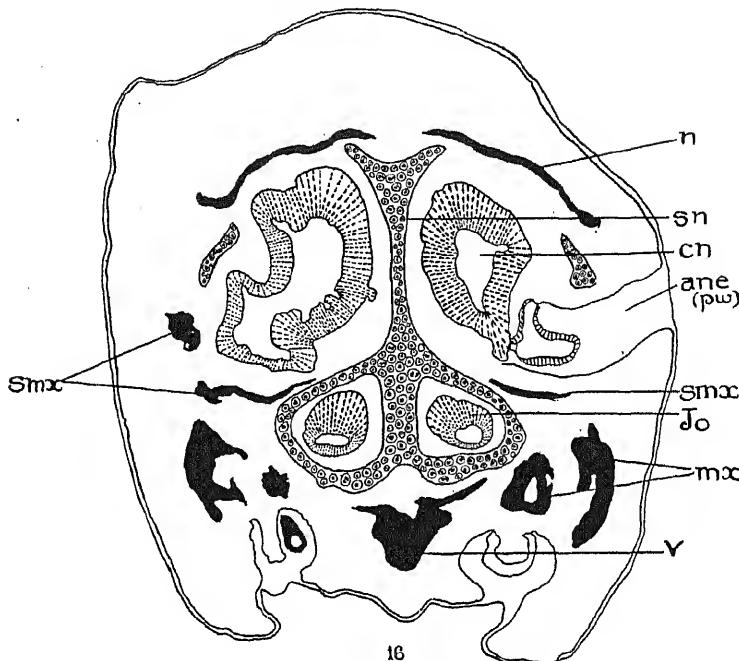
16   Transverse section through the nasal region of Sphenodon punctatus.
× 30.

17   Transverse section through the nasal region of Gonatodes vittatus.   × 40.
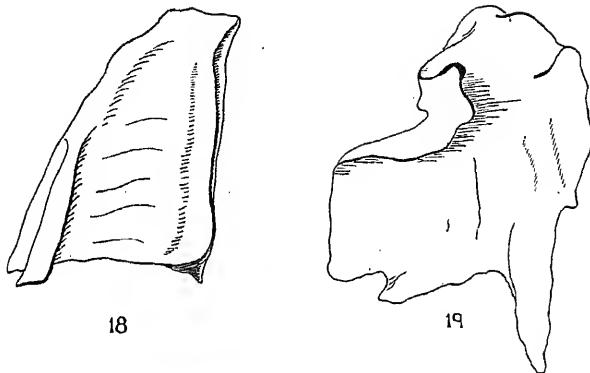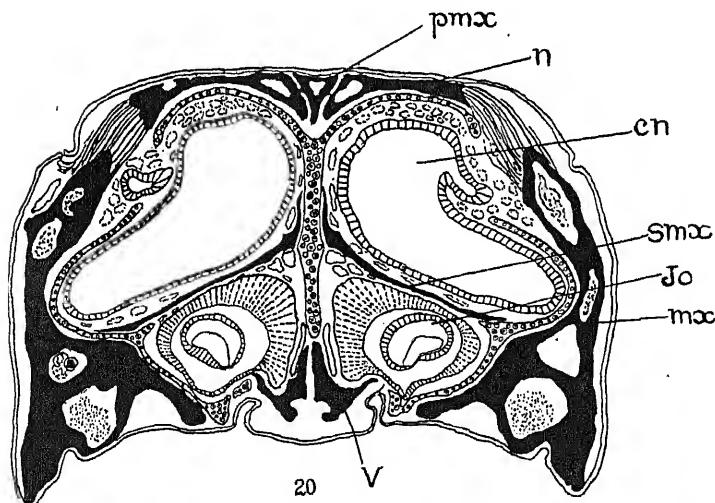
18   Left septomaxillary of G. vittatus, dorsal view of a reconstruction.   × 40.
19   Left septomaxillary of Lacerta muralis, dorsal view.   × 20.
20   Transverse section through the nasal region of L. muralis.   × 20.

PLATE 8

EXPLANATION OF FIGURES

21 Transverse section through the nasal region of Glauconia nigricans. × 30.

22 Left septomaxillary of G. nigricans, posterior view of a reconstruction. × 30.

23 Right septomaxillary of Lycodon aulicus, posterior view of a reconstruction. × 20.

24 Transverse section through the nasal region of L. aulicus. × 20.

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PLATE 8

SEPTOMAXILLARY OF THE ANURA AND REPTILIA. II

ENID OLDHAM LAPAGE
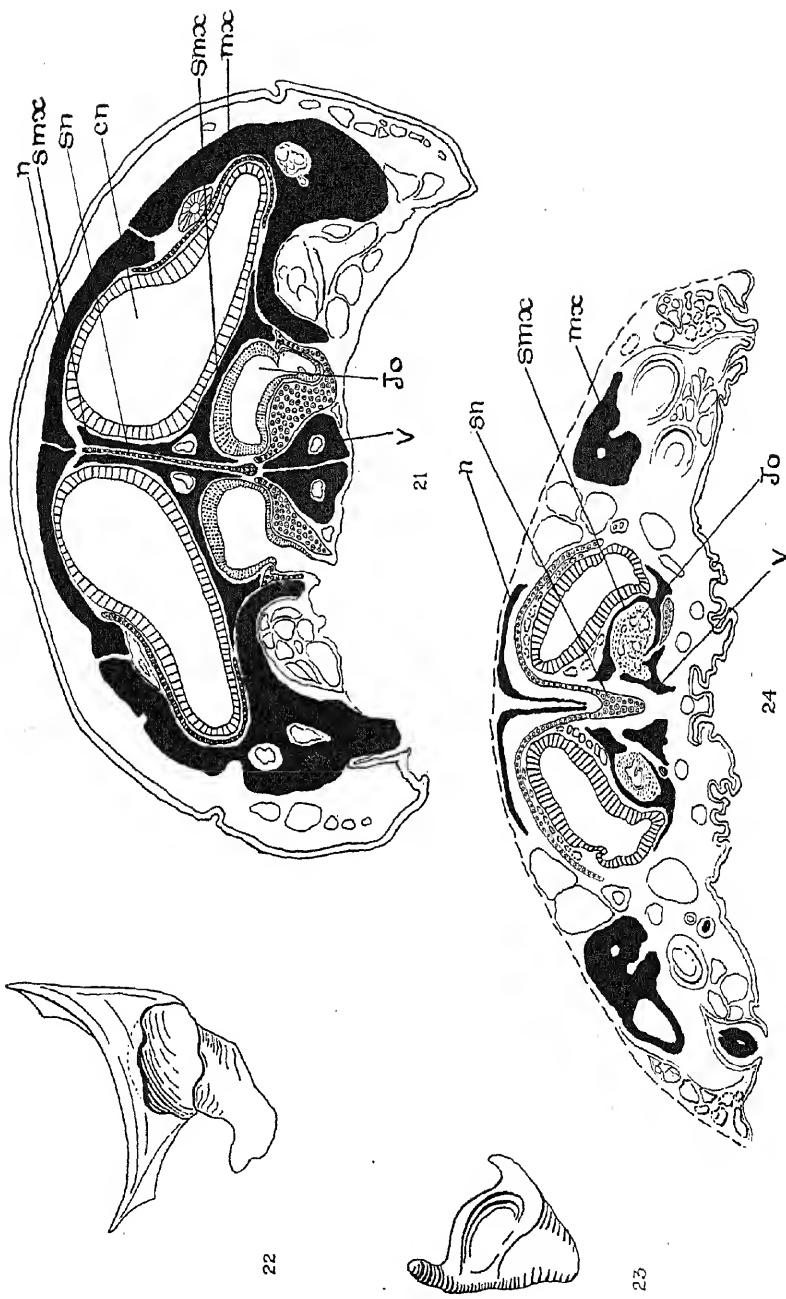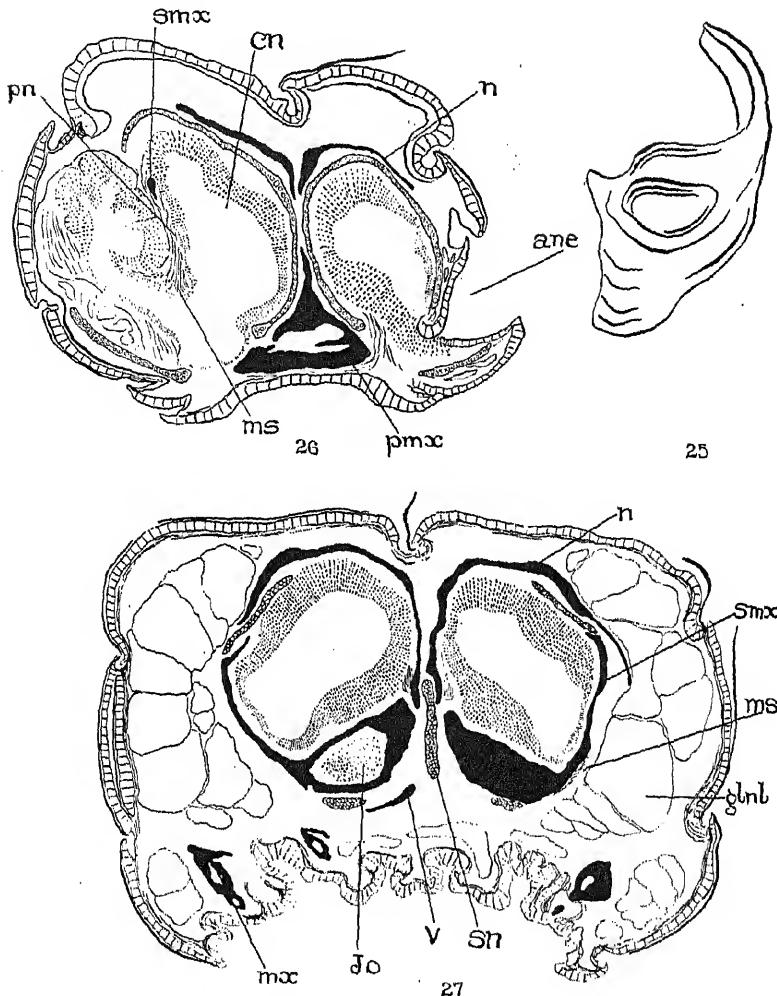
25 Right septomaxillary of Atractis trilineatus, left drawn in dots, posterior view of a reconstruction. × 40.

26 Transverse section through the region of the external nares of A. trilineatus. × 40.

27 Transverse section through the nasal region of A. trilineatus posterior to the nares. × 40.

ECOLOGICAL STUDIES OF THE SEASONAL DISTRIBUTION OF PROTOZOA IN A FRESH-WATER POND[1]

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EIGHT FIGURES

AUTHOR'S ABSTRACT

This study deals with the seasonal distribution of protozoa (February, 1927, to February, 1928) in correlation with seasonal fluctuations of temperature, hydrogen-ion concentration, and the relative amounts of dissolved oxygen and other gases in a small fresh-water pond. Twenty-seven species of Sarcodina, thirty-one species of Mastigophora, and 109 species of Infusoria were recorded in the surface water of the pond.

As found especially for Mastigophora and Infusoria, the number of species is inversely correlated with the abundance of individuals in the seasonal distribution.

Higher temperatures probably accelerated the rate of reproduction, since the seasonal maxima for most of the species were recorded in warmer weather.

Colonial flagellates and Zoochlorellae-bearing ciliates seemed to be favored by higher oxygen content, with a simultaneous abundance of volatile acids, especially CO_2. Although hydrogen-ion concentration was limited between 6.2 and 7.05, it was probably one of the factors influencing protozoan distribution, since several species disappeared when the lowest pH was recorded. Sunlight is one of the important factors in bringing certain heliotropic protozoa to the surface.

The seasonal maxima of many of the protozoa occurred during September and October, 1927, when most of the observed physical environments seemed much more favorable than in other months.

CONTENTS

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[1] A thesis in zoölogy presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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INTRODUCTION

In considering the distribution of the protozoa, it is a familiar fact that they may be found in all parts of the world under great variations in climate and other physical conditions of the environment. This might be expected, since they probably appeared on the earth at an early geological

age when the environment was different from what it is now, and a long evolutionary history has enabled them to become adapted to habitats of widely different sorts. However, the appearance and disappearance of particular species of protozoa in the same locality are usually correlated with seasonal changes, which indicates rather definite limitations of the optimal environment for them. It is therefore worth while to inquire into the question as to what effect changes in the physical environment may have on the seasonal distribution of protozoa.

The present study deals with the quantitative and qualitative analysis of the seasonal distribution of protozoa as affected by the seasonal fluctuations of temperature, hydrogen-ion concentration, and the relative amount of dissolved oxygen and other gases in a confined locality represented by a fresh-water pond located in the botanical gardens of the University of Pennsylvania.

In this investigation the author has been greatly indebted to Prof. David H. Wenrich, under whose guidance the present study was conducted, and to Prof. J. H. Bodine for many thoughtful suggestions offered during the progress of the work. He is also indebted to Dr. R. L. King for assistance on certain particular points.

METHODS OF STUDY

Description of the pond

As described by Calvert ('26), this artificial pond from which the material for study was obtained is oval in shape, covering an area of about 378 square meters. It is fed from the city water supply of Philadelphia. In warmer parts of the year, submerged vegetation of Chara, Elodea, Potamogeten, Nymphaea, and other plants attains a prolific growth in certain parts of the pond. Of metazoa, vertebrates consisting of goldfish (Carassius auratus), top-minnow (Gambusia affinis), sunfish (Eupomotis gibbosus), and bullfrog (Rana catesbiana), and invertebrates consisting of rotifers, ento-

mostracans, snails, and insect larvae, and other aquatic forms, are found in great abundance. Of particular interest here is the great number of species as well as individuals of protozoa which are to be found throughout the year, and it was to this group alone that attention has been given.

Collection of samples

A collecting station was established at a rock-bordered projection of the shore where the water was from 50 to 60 cm. deep. Collections were made at 9 A.M., three to five days a week, from February, 1927, to February, 1928. The water samples were always taken within a depth of 10 to 12 cm. beneath the surface. All floating masses were avoided in making collections, so that only planktonic samples were taken. Two liters of the water were procured in a porcelain pitcher for protozoological examinations. The samples for chemical tests were collected in a pyrex-glass flask, which was immediately tightly stoppered. Both the air temperature and water temperature were taken and recorded. The conditions of the weather were also briefly noted.

Chemical tests

The oxygen content of the water was determined by the micro-Winkler method given by Birge and Juday ('11, '22).

The hydrogen-ion concentration, expressed as pH value, was determined colorimetrically, at room temperature (23°C. to 25°C.) immediately after collections, using di-bromo-ortho-cresol-sulphon-phthalein, di-bromo-thymol-sulphon-phthalein, phenol-sulphon-phthalein, and ortho-cresol-sulphon-phthalein as indicators.

After the pH of the fresh sample was taken, the rest of the pond water in a bottle was boiled for ten minutes and the pH taken again at room temperature after cooling. The difference between the pH of the fresh sample and that after boiling was assumed to be the result of the loss of volatile acids, such as CO_2, H_2S, and traces of others, which had been previously dissolved in the sample under natural conditions.

By this method the data were obtained by which seasonal variations of the relative amount of these gases present in the water could be approximately determined.

Quantitative determination of protozoan distribution

For quantitative determination of the protozoan population, the Sedgwick-Rafter method (Whipple, '27) was employed. In every case 500 cc. of water sample was filtered through a layer of sand in a graduated cylindrical funnel. The detained animals were washed from the sand and, after separating the concentrate from the sand by decantation, some of it was introduced into a counting cell. With the aid of an ocular disk ruled into squares, all the species of protozoa were enumerated in twenty squares, and from the resulting value the number of each species present in 1 cc. of a sample was computed.

In the operation of this method, many delicate protozoa were often observed to be undergoing disintegration or deformation in the counting cell. In order to check discrepancies arising from such losses, enumerations of the protozoan population were also made from another sample of a measured quantity which had been concentrated by ordinary filtration with filter-paper.

Arrangement of data

All the information thus gained for each day was entered in one record sheet, and at the end of the work the daily notes were combined into results by weeks. In taking the weekly average, standard deviation of the individual numbers for each species was made.

Finally, the weekly changes in physical environment and in protozoan population were arranged into a large table, where the results were conveniently available for further study and analysis. Since this table is about 32 inches in width and 68 inches in length, it is too cumbersome to be published. Therefore, a list is made of all observed species with brief notes on their distribution. Like the data on physical en-

vironment, those for species of particular interest are represented by graphs.

OBSERVATIONS

A. *List of protozoa observed, with notes on their seasonal distribution*

Sarcodina:

Actinophrys sol Ehr.: very rare, July 4 to 24 and September 19 to 25.

Actinosphaerium eichhornii Ehr.: number small, occasionally in all seasons; maximum, September 5 to 11.

Amoeba discoides Schaef.: number small, May 30 to June 24 and September 5 to November 27; maximum, October 10 to 16.

Amoeba guttula Duj.: very rare, September 19 to 23 and January 16 to 22.

Amoeba limax Duj.: very rare, May 30 to June 5.

Amoeba polypodia Schulze: see chart 3, graph 2.

Amoeba striata Penard: very rare, October 3 to 16.

Amoeba verrucosa Ehr.: very rare, July 18 to 31.

Arcella discoides Ehr.: .01 to .22 per cc., May 30 to October 30; maximum, August 1 to 14.

Arcella vulgaris Ehr.: number small, July 18 to October 16.

Centropyxis aculeata Stein: number small, June 6 to November 6.

Centropyxis ecornis Leidy: number small, occasionally in spring and summer.

Clathrulina elegans Cienk.: .02 to .24 per cc., September 5 to October 9 and November 7 to 13; maximum, September 12 to 18.

Dactylosphaerium radiosum (Ehr.): number small, April 11 to 17, July 18 to 31, and November 7 to 13.

Difflugia acuminata Ehr.: very rare, occasionally in spring and early fall.

Difflugia constricta Ehr.: number small, March 21 to April 3 and June 6 to July 10; maximum, June 13 to 19.

Difflugia corona Wall.: .008 to .180 per cc., May 23 to June 26 and July 25 to December 11.

Difflugia lobostoma Leidy: see chart 3, graph 1.

Difflugia pyriformis Perty: same as D. acuminata.

Euglypha alveolata Duj.: .008 to .140 per cc., March 14 to July 17; maximum, April 18 to 24.

Gromia fluviatilis Duj.: .01 to .52 per cc., May 9 to 22, July 25 to 31, and September 12 to October 2; maximum, September 26 to October 2.

Nebela collaris Leidy: very rare, June 6 to 12.

Nebela dentistoma Penard: see chart 3, graph 3.

Nuclearia simplex Cienk.: very rare, June 13 to 19 and January 9 to 15.

Pamphagus hyalinus Ehr.: very rare, January 23 to 29.

Raphidiophrys elegans H. and L.: number small, occasionally in all seasons; maximum, September 12 to 18.

Vampyrella sp.?: very rare, February 14 to 20, March 14 to 20, and May 30 to June 5.

Mastigophora:

Chlamydomonas pulviculus Ehr.: .3 to 10.0 per cc., February 7 to April 17
and January 2 to February 5; maximum, February 28 to March 6.

Cryptomonas erosa Ehr.: .008 to .220 per cc., occasionally in all seasons;
maximum, January 9 to 15.

Dinobryon sertularia Ehr.: see chart 5, graph 1A.

Entosiphon sulcatus Stein: number small, March 14 to 27.

Eudorina elegans Ehr.: see chart 5, graph 2B.

Euglena acus Ehr.: very rare, May 3 to 29.

Euglena deses Ehr.: number small, occasionally in March 28 to October 23;
maximum, July 11 to 17.

Euglena mutabilis Schmitz: very rare, May 9 to 29.

Euglena oxyuris Schmarda: very rare, July 25 to August 7.

Euglena terricola Duj.: very rare, July 25 to 31.

Euglena viridis Ehr.: see chart 4, graph 1.

Glenodinium cinctum Ehr.: see chart 5, graph 2A.

Gonium pectorale Mull.: .02 to 2.0 per cc., February 7 to May 8 and
August 22 to February 5; maximum, November 28 to December 4.

Gymnodinium aeroginosum Stein: number small, March 21 to April 17.

Heteronema acus Ehr.: very rare, July 11 to 17.

Leptocinclis ovum Ehr.: .008 to .150 per cc., March 7 to June 5 and July 4
to August 14; maximum, April 18 to 24.

Menoidium pellucidum Perty: number small, April 4 to 24, July 18 to 31,
August 22 to 28, and September 22 to 28.

Notosolenus orbicularis Stokes: rare, March 7 to 20 and April 11 to 17.

Pandorina morum B. de S. V.: number small, occasionally in spring and fall.

Peranema trichophorum Ehr.: .004 to .110 per cc., March 22 to 27, May 30
to August 21, and September 5 to October 23; maximum, September 26
to October 2.

Peridinium cinctum Ehr.: see chart 5, graph 1B.

Petalomonas mediocanellata Stein: very rare, March 7 to 13.

Phacus alata Klebs: very rare, July 18 to 24.

Phacus anacoelus Stokes: .01 to .38 per cc., July 18 to October 30.

Phacus longicaudatus Ehr.: .004 to .660 per cc., March 14 to 20, April 11
to May 8, May 30 to July 3, and July 11 to October 16; maximum,
July 25 to 31.

Phacus pleuronectes Nitze: see chart 4, graph 3.

Synura uvella Ehr.: see chart 5, graph 3.

Trachelomonas armatus Ehr.: .008 to .170 per cc., April 18 to July 3 and
July 25 to October 9; maximum, June 13 to 19.

Trachelomonas hispida Stein: see chart 4, graph 2.

Trachelomonas urceolata Stokes: number small, occasionally in July 18 to
October 9.

Uroglena americana Calkins: .01 to 3.80 per cc., April 25 to May 22;
maximum, May 9 to 15.

Infusoria:

Acineta fluviatilis Stokes: rare, August 22 to 28.

Actinobolus radians Stein: .01 to 1.30 per cc., May 9 to 15, July 18 to August 7, and August 22 to September 4; maximum, May 9 to 15.

Amphileptus sp.?: rare, August 15 to 21 and November 21 to 27.

Arachnidium bipartitum From.: see chart 6, graph 5B.

Arachnidium globosum Kent: number small, occasionally in summer.

Askenasia elegans Bloch.: .008 to 4.6 per cc., June 20 to November 6; maximum, July 4 to 10.

Aspidisca costata Duj.: see chart 7, graph 2.

Aspidisca turrita C. and L.: number small, January 19 to 29.

Campanella sp.?: see chart 7, graph 3.

Carchesium polypinum Linn.: rare, February 7 to 13.

Chaenia teres Duj.: very rare, January 9 to 15.

Chilodon caudatus Stokes: rare, July 18 to 24.

Chilodon cucullulus Mull.: .08 to 1.6 per cc., March 7 to April 3, May 2 to 8, May 30 to June 5, October 24 to 30, November 14 to December 18, and January 2 to 8; maximum, March 14 to 20.

Chilodon megalotrochae Stokes: rare, May 2 to 8.

Chilodon vorax Stokes: .01 to .40 per cc., May 30 to June 5 and September 19 to October 23.

Cinetochilum margaritaceum Ehr.: .08 to 3.0 per cc., April 4 to December 4 and January 9 to February 5; maximum, October 3 to 9.

Coleps elongatus Ehr.: .03 to 11.2 per cc., January 2 to February 5; maximum, January 30 to February 5.

Coleps hirtus Nitzsch: see chart 6, graph 1.

Colpoda campyla Stokes: .007 to 1.9 per cc., June 13 to 26, October 10 to November 13, and December 12 to January 22; maximum, October 10 to 16.

Colpoda cucullulus Ehr.: rare, February 7 to 27 and April 18 to 24.

Craspedonotus vermicularis (Kahl): .008 to .9 per cc., April 25 to May 1, September 26 to October 2, October 24 to 30, and November 14 to December 18; maximum, April 25 to May 1.

Cyclidium glaucoma Ehr.: see chart 6, graph 4B.

Cyclotrichium vernalis Wenrich: .02 to 10.7 per cc., April 25 to May 22; maximum, May 9 to 15.

Dallasia frontata Stokes: rare, March 7 to 20, September 5 to October 9, and November 7 to 13.

Dexiotricha plagia Stokes: rare, July 18 to 24.

Didinium nasutum Mull.: see chart 8, graph B.

Dileptus gigas C. and L.: .07 to 2.1 per cc., July 11 to August 21, October 3 to 9, November 7 to 27, and January 2 to 22; maximum, November 14 to 20.

Enchelys sp.?: number small, May 30 to June 10.

Epistylis plicatalis Ehr.: rare, September 5 to 18.

Euplotes charon Mull.: number small, November 7 to December 11.

Euplotes patella Ehr.: see chart 7, graph 1B.

Euplotes plumipes Stokes: .008 to 1.4 per cc., July 4 to November 20; maximum, October 24 to 30.

Infusoria (continued):

Frontonia acuminata C. and L.: see chart 6, graph 4A.

Frontonia leucas C. and L.: .008 to .8 per cc., March 14 to April 17, June 20 to 26, August 8 to 14, August 29 to October 30, and January 2 to February 5; maximum, October 3 to 9.

Glaucoma scintillans Ehr.: rare, November 14 to 20.

Halteria grandinella Mull.: see chart 6, graph 2.

Histrio erethisticus Stokes: number small, April 18 to 24, June 13 to July 3, and November 14 to 20.

Holophrya gargamella F. F.: see chart 6, graph 3A.

Holophrya kessleria Mereschk: see chart 6, graph 3B.

Holophrya sp.?: .1 to 6.3 per cc., April 18 to June 5, July 18 to August 14, and January 16 to 22; maximum, April 25 to May 1.

Holosticha vernalis Stokes: number small, June 27 to July 17, October 17 to 23, December 5 to 11, and December 26 to January 1.

Kerona pediculus Mull.: rare, March 14 to 20 and October 17 to 23.

Lacrymaria phyalina Svec.: .2 to 1.9 per cc., occasionally in spring and summer; maximum, April 18 to 24.

Lagynus elegans Schew.: very rare, May 30 to June 5.

Lembadion bullinum Perty: .01 to 25.0 per cc., March 21 to 27, April 18 to May 8, and August 15 to October 30.

Lionotus fasciola Wrze.: .07 to 2.9 per cc., occasionally in February, April, and January, frequently in May 16 to November 13; maximum, October 3 to 9.

Lionotus pleurosigma Stokes: .004 to 1.2 per cc., occasionally in May 30 to July 10, frequently in October 3 to November 27; maximum, October 31 to November 6.

Lionotus wrzesniowskii Kent: .07 to 2.1 per cc., March 21 to 27, April 18 to May 1, May 23 to June 5, and August 8 to November 20; maximum, October 3 to 9.

Loxodes rostrum Mull.: .01 to 1.2 per cc., August 15 to November 20; maximum, October 10 to 16.

Loxophyllum lamella Ehr.: number small, occasionally in September, October, and January.

Loxophyllum meleagris Ehr.: number small, March 14 to 20, May 16 to 24, and August 1 to October 16.

Monodinium balbianii F. D.: .8 to 9.3 per cc., April 18 to May 15; maximum, April 25 to May 1.

Opercularia berberina Linn.: rare, September 19 to 25.

Opercularia stenostoma Stein: .68 per cc., April 4 to 10.

Ophrydium versitale Mull.: rare, April 11 to 17.

Oxytricha bifaria Stokes: .004 to .5 per cc., May 23 to June 5, and occasionally in October 24 to January 1; maximum, May 30 to June 5.

Oxytricha fallax Stein: rare, June 20 to 26 and January 9 to 22.

Oxytricha pellionella Mull.: see chart 7, graph 1A.

Oxytricha platystoma Ehr.: .008 to 3.4 per cc., August 1 to September 25; maximum, September 12 to 18.

Paramecium aurelia Mull.: rare, June 27 to July 3 and October 17 to 23.

Infusoria (continued):

Paramecium bursaria (Focke): see chart 6, graph 5A.

Paramecium caudatum Ehr.: see chart 8, graph A.

Paramecium trichium Stokes: .008 to .16 per cc., April 18 to 24, May 16 to June 5, June 27 to July 10, August 15 to 21, August 29 to September 11, and occasionally in October to February; maximum, May 23 to 29.

Phascolodon vorticella Stein: rare, May 2 to 8 and 16 to 22.

Plagiopyla nasuta Stein: rare, October 3 to 9.   .

Pleuronema chrysalis Ehr.: .01 to .2 per cc., January 9 to 22.

Pleurotricha lanceolata Ehr.: number small, February 7 to 13 and January 2 to 22.

Prorodon griseus C. and L.: .08 to 6.4 per cc., March 7 to 20, April 4 to May 8, May 16 to 22, June 6 to 12, and July 4 to October 2; maximum, July 25 to 31.

Prorodon margaritifera (?) C. and L.: .1 to 1.7 per cc., July 11 to August 14; maximum, August 1 to 7.

Rhabdostyla invaginata Stokes: .24 to 8.6 per cc., May 30 to July 3; maximum, June 20 to 26.

Rhabdostyla vernalis Stokes: 2.2 per cc., June 13 to 19.

Spirostomum ambiguum Ehr.: very rare, May 9 to 15.

Spirostomum teres C. and L.: .01 to 2.5 per cc., July 25 to October 30; maximum, September 5 to 11.

Stentor amethystinus Leidy: .004 to .5 per cc., February 21 to 27 and January 2 to February 5; maximum, January 16 to 22.

Stentor barretti Barrett.: .008 to 2.4 per cc., August 1 to December 18; maximum, November 14 to 20.

Stentor coerulens Ehr.: .008 to 4.2 per cc., July 25 to November 6; maximum, September 5 to 11.

Stentor igneus Ehr.: .01 to 2.4 per cc., July 4 to September 11; maximum, August 1 to 7.

Stentor polymorphus Mull.: .008 to 1.1 per cc., occasionally in February to August and January, frequently in September 5 to December 18; maximum, May 23 to 29.

Stentor roseus From.: .008 to .8 per cc., March 7 to May 1 and November 21 to February 5; maximum, January 30 to February 5.

Stichotricha secunda Perty: rare, May 30 to June 5, June 27 to July 3, and August 8 to 14.

Strombidium claparèdii Kent: see chart 6, graph 6A.

Strombidium viridis Stein: .008 to 2.2 per cc., February 7 to May 15 and December 19 to January 29; maximum, February 28 to March 6.

Strombilidium gyrans Stokes: .07 to 13.4 per cc., frequently in all seasons except January 9 to February 5; maximum, October 3 to 9.

Stylonychia mytilis Mull.: .008 to .24 per cc., March 21 to 27, May 16 to June 5, June 20 to July 10, September 25 to November 13, and January 9 to 29; maximum, May 23 to 29.

Stylonychia notophora Stokes: .02 to 1.3 per cc., May 23 to June 5 and July 11 to 17; maximum, May 23 to 29.

Infusoria (continued):

Stylonychia pustulata Ehr.: .004 to 1.3 per cc., February 7 to 27, May 23 to June 5, June 20 to July 10, October 24 to November 13, and January 9 to February 5; maximum, January 16 to 20.

Tachysoma parvistyla Stokes: .002 to .6 per cc., occasionally in spring, frequently in November 7 to February 5.

Teuthophrys trisulca C. and B.: see chart 6, graph 7.

Thuricola valvata Wright: rare, September 26 to October 2.

Trachelius ovum Ehr.: number small, occasionally in spring and fall.

Trachelius subtilis Penard: .1 to 2.1 per cc., March 14 to 26, May 2 to 15, May 30 to June 5, July 4 to October 16, and October 31 to December 11; maximum, September 19 to 25.

Trichodina sp.?: rare, April 11 to 17, May 1 to 15, and July 11 to 17.

Urocentrum turbo Mull.: see chart 6, graph 6B.

Uroleptus dispar Stokes: .007 to .6 per cc., May 23 to June 5, July 4 to August 21, and November 21 to December 18.

Uroleptus longicaudatus Stokes: number small, occasionally in June and July.

Uroleptus musculus Mull.: number small, occasionally in March, June, October, and December.

Uroleptus piscis Mull.: .005 to .6 per cc., February 7 to 20, July 18 to August 21, and October 24 to December 11; maximum, November 28 to December 4.

Urostyla grandis Ehr.: rare, occasionally in September 5 to January 22.

Vaginicola crystallina Ehr.: rare, May 30 to June 5 and June 27 to July 3.

Vaginicola leptosoma Stokes: .008 to 1.2 per cc., occasionally in March 21 to July 17 and September 26 to October 30; maximum, May 2 to 8.

Vaginicola longicollis But.: number small, March 21 to April 3.

Vorticella aperta From.: rare, March 28 to April 3.

Vorticella campanula Ehr.: .08 to 18 per cc., frequently in March 28 to November 27; maximum, June 20 to 26.

Vorticella crassicaulis Kent: .3 to 2.1 per cc., February 7 to March 13 and March 28 to April 17; maximum, March 28 to April 3.

Vorticella microstoma Ehr.: .4 to 2.1 per cc., May 30 to July 17 and November 7 to 13; maximum, June 20 to 26.

Vorticella nebulifera Ehr.: .08 to 3.3 per cc., frequently in April 25 to November 20, traces in January 2 to February 5.

Vorticella nutans Mull.: rare, April 11 to 17.

Zoothamnium aselli C. and L.: rare, January 23 to 29.

Zoothamnium simplex Kent: rare, February 7 to 13 and June 20 to 26.

B. Observations on the physical environment

The following presentation of observed data involves only a part of the extrinsic factors related to the seasonal distribution of protozoa. Intrinsic factors have not been dealt with. As a result of the reproductive activities, protozoan

life is primarily cyclical in character, so that the seasonal appearance and disappearance of a species may not be entirely due to the changes of the external environment. In other words, the activities of a protozoan cell at any time must be recognized as a resultant of the entire series of factors which operate simultaneously inside as well as outside of the animal.

For convenience, each of the environmental factors studied will be considered separately, but in pointing out the correlations between changes in any factor and the variations in numbers of the protozoa, it is always recognized that no one factor is necessarily acting alone.

Temperature. The data for seasonal variations in the factors of the physical environment are entered in chart 1. Graph 1 illustrates the variations in temperature. As the graphs show, the air and water temperatures are never exactly the same, but the differences are slight, varying between 0.1°C. (August 29th to September 4th) and 3.6°C. (December 5th to 11th). The way in which the fluctuations of water temperatures are directly influenced by those of the air is, however, fairly well indicated. It should be noted that the data on observed air temperature have been compared with the mean daily temperatures obtained from the U. S. Weather Bureau in Philadelphia. These parallel one another very closely, with an average difference of less than 2°C. Since my data on the observed thermal changes were made at 9 A.M. in every case, it would seem that the air temperature of that particular time of the day may rather safely represent the daily mean temperature.

The seasonal range of temperature is from $-3.5°$C. to $26.3°$C. for air and $1.1°$C. to $25.9°$C. for water. Both of the lowest temperatures were recorded in the week of January 30th to February 5th and those of the highest were observed during the week of July 11th to 17th. The thermal changes fluctuated rather regularly in most parts of the year. In the week of March 14th to 20th there was an unusual increase of temperature of approximately 6°C. above that of the pre-

ceding and succeeding weeks. Similar unexpected changes in temperature were also recorded for five successive weeks from November 14th to December 18th.

Oxygen content. As was to be expected, the graph for dissolved oxygen (graph 2, chart 1) runs, as a whole, in an opposite direction to those for temperature. It shows that, in late summer and early fall, the water contains less oxygen and the amount tends to increase gradually as the temperature decreases. This accords with the known fact that the amount of dissolved gases in water is inversely proportional to the temperature. Direct correlations may be noted in several instances. In July 11th to 17th, when the temperature reached the highest in the year, the oxygen content dropped to its minimum. In March 14th to 20th, a sudden increase of temperature is associated with a correspondingly marked depletion in oxygen content. The highest oxygen content was recorded in the weeks of December 19th to 25th and January 2nd to 8th, when the thermal range was very low.

It is generally accepted that water at $0°C$. may hold 10.29 cc. of oxygen per liter, as against 6.57 cc. at $20°C$. On the other hand, the data on the observed amount of oxygen in this pond cover a very wide seasonal range from 2.3 cc. to 15.3 cc. per liter. This leads us to believe that there are causal factors other than temperature for the concentration of dissolved oxygen. The excessive amounts are probably due to the photosynthetic process of the green plants. It is of interest to note that the readings on oxygen content taken on cloudy or rainy days were usually lower than those made on sunny days regardless of the slight changes in temperature. The presence of large numbers of oxygen-consuming organisms in the pond during the late summer and early fall may have diminished the oxygen content to a certain extent beyond the limits set by the thermal changes.

Hydrogen-ion concentration. The hydrogen-ion concentration of the pond water is fairly constant throughout the year, with a narrow range of pH between 6.2 and 7.05 (graph 3, chart 1). In spite of its small fluctuations, the pH change
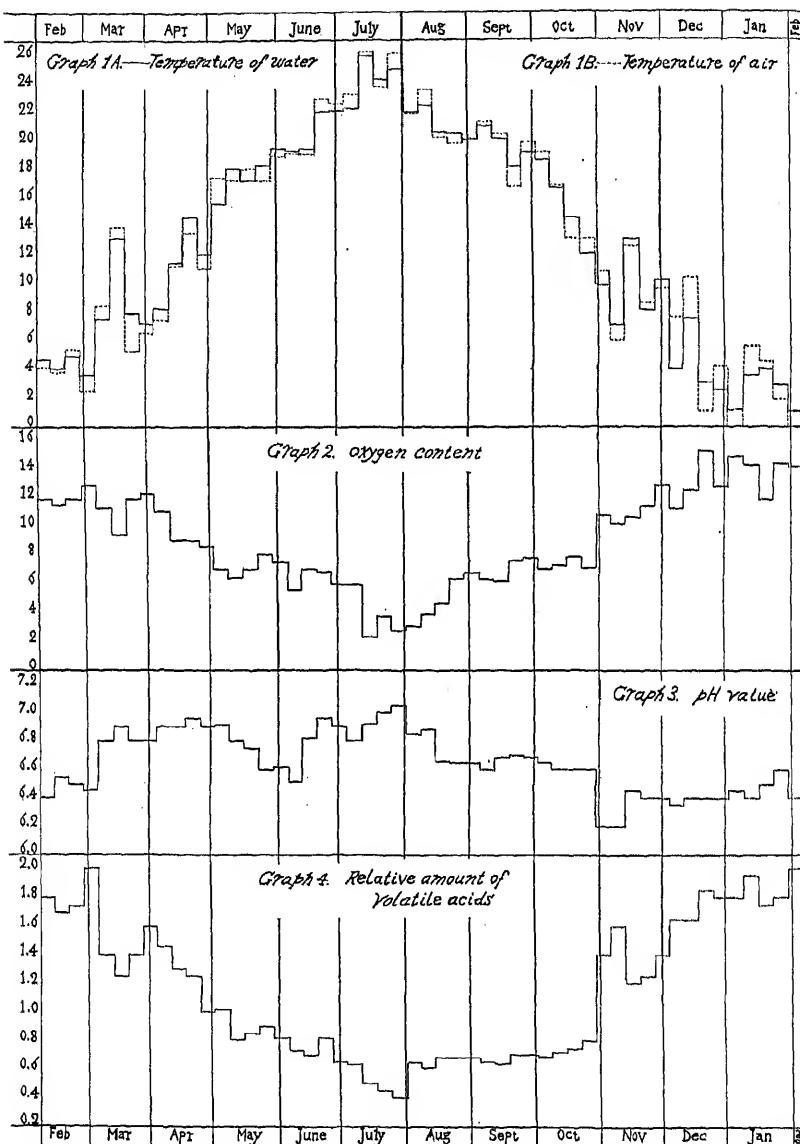
Chart 1 Seasonal variations of physical environments. Graph 1A, water temperature; 1B, air temperature, centigrade scale. Graph 2, oxygen content in cubic centimeters per liter. Graph 3, pH value. Graph 4, relative amounts of volatile acids, ordinates showing degree of difference between the pH of fresh sample and that after boiling.

is indirectly correlated with the seasonal changes of temperature. It will be noted that the readings of high pH are usually found at higher temperature and those of low pH at lower temperature.

Volatile acids. As in the case of oxygen, the graph (graph 4, chart 1) for the degree of difference between the pH of the fresh sample and that after boiling tends to run in a reverse manner in comparison with the temperature. As previously noted (pp. 434, 435), this operation was undertaken in order to determine the relative amounts of volatile acids dissolved in the water. The results again are in accordance with the common rule that the lower the temperature of water, the larger the amount of gas which will dissolve, and vice versa.

Of the volatile acids, carbonic-acid gas probably represents the greatest proportion. The presence of hydrogen sulphide was often confirmed by its peculiar odor. Traces of other volatile acids which may lower the pH value probably also occur in this pond.

Correlations between the pH changes and the relative amount of volatile acids in water are well illustrated in the weeks of March 14th to 20th, July 25th to 31st, and October 31st to November 13th. But this relation, as a whole, did not hold at all times, since the range of seasonal variation of the difference between the two pH readings may be several times as great as the range of the original pH of the fresh sample (graphs 3, 4, chart 1). It seems possible that the pond water also contained a certain amount of dissolved carbonates of calcium or magnesium acting naturally as buffers to keep the pH approximately constant, even if the quantitative changes of the dissolved acids in water are large.

C. Seasonal distribution of protozoa according to groups

In chart 2 three graphs illustrate the seasonal distribution of Sarcodina, Mastigophora, and Infusoria, respectively. In comparing these graphs with the number of species in the

list, one will easily understand the nature of the relative
abundance and diversity as represented by these three forms.
In the Sarcodina, only twenty-seven species have been ob-
served, and the total number of individuals of various species
is likewise small, with the highest population just over
thirteen per cubic centimeter. Of Mastigophora, thirty-one
species were found, while the number of individuals was the
greatest among the groups. With 109 species, the Infusoria
show the greatest diversity, but the maximum abundance of
the total individuals per cubic centimeter is only 921 as
against 1331 in the case of the Mastigophora.

Sarcodina. Most of the Sarcodina are creeping organisms
and usually inhabit the bottom of the pond. It is not sur-
prising that the number of both species and individuals is
very limited, since the collections were made from the sur-
face water. As the data stand, however, their seasonal popu-
lation fluctuated in a manner similar to the other forms. The
numbers tended to increase as the temperature rose in the
spring and decreased in the fall as the temperature dropped.
Including two well-defined maxima, the period of greatest
abundance of Sarcodina occurred between August 1st and
October 16th, when the ranges of their physical environments
are: temperature, 16.7°C. to 22.4°C.; oxygen content, 3.05 to
7.76 cc. per liter; pH, 6.6 to 6.88, and a low amount of volatile
acids (compare with chart 1).

Mastigophora. In considering the seasonal variation in
the population of Mastigophora, one will notice the rough-
ness of the graph represented in chart 2. As will be discussed
later, each of the outstanding peaks in the graph is due to
a rapid increase in individuals of certain species. The maxi-
mum abundance of Dinobryon sertularia, Eudorina elegans,
Peridinium cinctum, and Glenodinium cinctum occurring at
various times of the year is the main factor in producing the
irregularities in the graph (compare with graphs 1A, 1B,
2A, 2B, chart 4). Thus the two largest maxima of popula-
tion of Mastigophora, one in winter (February 14th to 22nd)
and another in late summer (August 22nd to 28th) resulted

from the great numbers of Dinobryon sertularia and Peri-
dinium cinctum, respectively. It will be understood, then,
that the Mastigophora were primarily represented by two
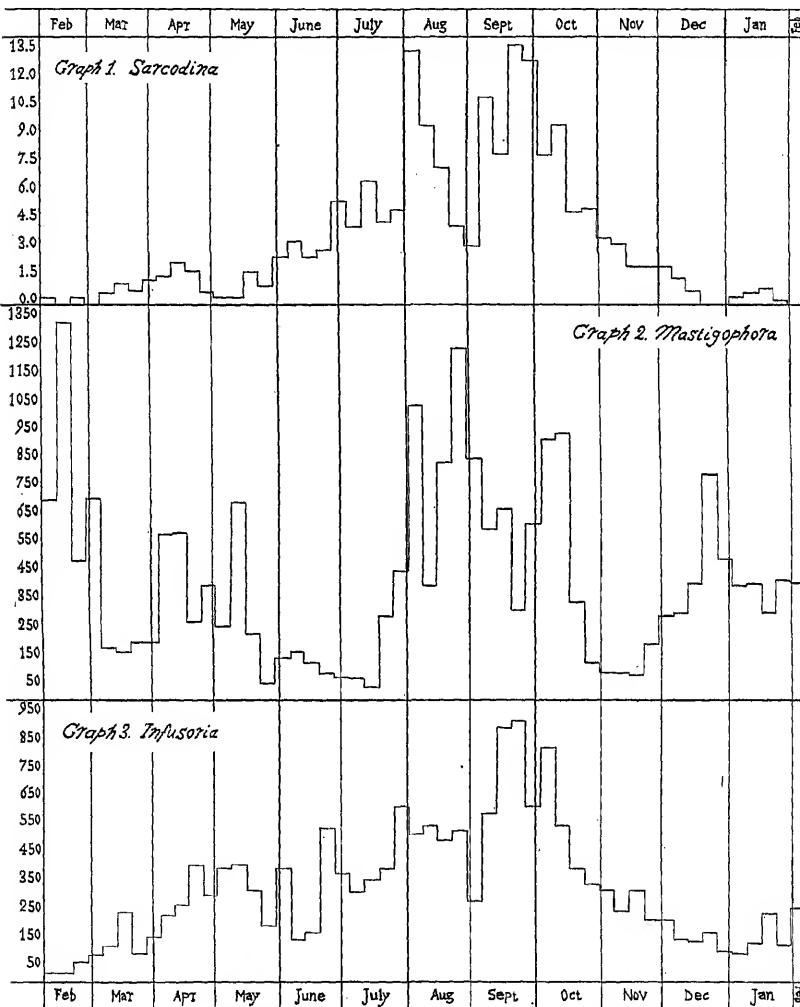
Chart 2 Showing the seasonal distribution of Sarcodina (graph 1), Masti-
gophora (graph 2), and Infusoria (graph 3). Ordinates indicate the number of
animals per cubic centimeter.

different forms which were favored by environments of extremely divergent sorts. Therefore, it seems impossible to determine in any adequate way the relation of the whole group to the changes of their surroundings.

Infusoria. The graph for the seasonal distribution of Infusoria is less irregular than the graphs for Sarcodina and Mastigophora. As with the Sarcodina, the seasonal variations in the population of Infusoria are well correlated with the fluctuations of temperature. On the other hand, there is only one maximum for the Infusoria found between September 12th and 25th, when the temperature was not the highest. If high numbers of Infusoria are correlated with high temperature, then there is a lag in the effect on the population, since the highest temperature was recorded several weeks before September 12th. (A similar lag may be true for Sarcodina and dinoflagellates.)

Since the Infusoria are represented by the greatest number of species and since individual species require different optimal ranges in the physical environment, it is difficult to establish correlations between the latter and the whole group. At best, it may be pointed out that, excluding the case of the weeks, June 20th to 26th and August 29th to September 4th, the individual numbers of Infusoria were over 500 per cubic centimeter from July 25th to October 16th, when the fluctuations of physical environment were: temperature, 16.7°C. to 25.8°C.; oxygen content, 2.77 to 7.6 cc. per liter; pH, 6.6 to 7.05, and lower amount of volatile acids.

D. *Correlation between temperature and the seasonal distribution of protozoa*

Sarcodina. The majority of the species of Sarcodina studied have wide ranges of toleration for thermal changes and, therefore, may be grouped as eurythermal animals. The quantitative analysis indicates, however, that they tend to thrive better in warmer water than in colder, at least as they appear in the surface water.

The various species of Amoeba were never found in extremely cold water, except in the case of A. guttula (January 16th to 22nd). The frequent occurrence of A. polypodia (graph 2, chart 3) from May 30th to October 30th exhibits an interesting correlation with the optimal range of temperature. It lives within the thermal range of 12°C. to 25°C.,
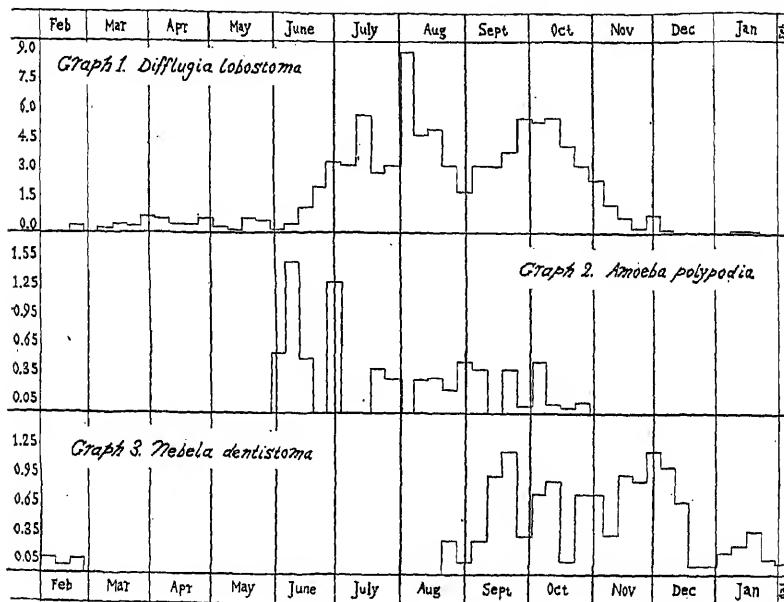
Chart 3   Showing the seasonal distribution of Difflugia lobostoma (graph 1), Amoeba polypodia (graph 2), and Nebela dentistoma (graph 3).   Ordinates indicate the number of animals per cubic centimeter.

with its maximum abundance under 19.3°C., and it should be thus considered as stenothermal, rather than eurythermal.

The seasonal distribution of Difflugia lobostoma (graph 1, chart 3) showed a gradual increase of population toward the higher temperatures.   Immediately after the highest temperature in the latter part of July, the population of D. lobostoma reached its maximum in the week of August 1st to 7th.   A long-continued minor abundance (a short one in the week of July 11th to 17th) from September 26th to

October 13th is probably correlated with the unusually high
temperatures in October, 1927. Other Testacea, like species
of Arcella and Centropyxis and Euglypha alveolata, also
tend to be more abundant in the higher temperatures.

Nebela dentistoma (graph 3, chart 3) seems to be favored
by cold water, since it appeared between 1.1°C. to 21°C. and
has never been observed during summer. There were, how-
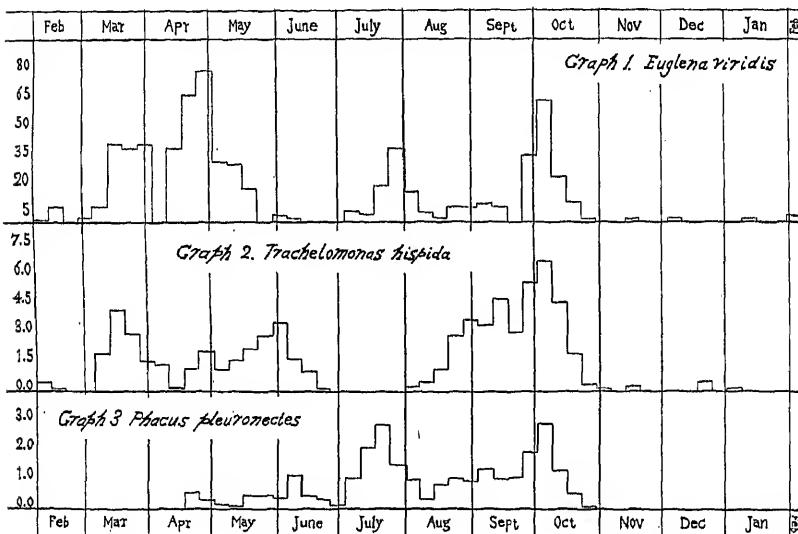
Chart 4 Showing the seasonal distribution of three common flagellates,
Euglena viridis (graph 1), Trachelomonas hispida (graph 2), and Phacus
pleuronectes (graph 3). Ordinates indicate the number of animals per cubic
centimeter.

ever, two maxima for this species at quite different tempera-
tures, one at 18.1°C. and another at 10.2°C.

Mastigophora. The optimum range of temperature for
individual species of Mastigophora is as varied as in the case
of Sarcodina. Euglena viridis (graph 1, chart 4), as ob-
served in all parts of the year, may live within the thermal
range between 1.1°C. and 25.9°C. Three unequal maxima for
E. viridis were found in the latter parts of spring, summer,
and autumn, and the temperature for these was 12°C., 22.1°C.,

and 18.6°C., respectively. The population in winter was exceedingly small. In the week of March 14th to 20th, when the temperature suddenly increased, there was a corresponding increase in individuals of E. viridis. Such correlations are, however, not found in other parts of the year, so that there are doubtless factors other than temperature that govern the distribution of this particular species.

Like E. viridis, Peridinium cinctum and Glenodinium cinctum represent the same eurythermal type in regard to their distribution. On comparing the graphs (graphs 1B, 2A, chart 5) for their seasonal variations with those for the fluctuations of temperature, one will notice that the individuals of P. cinctum were constantly over 300 per cubic centimeter at temperatures from 20.1°C. to 25°C. and those of Glenodinium cinctum were over 200 per cubic centimeter between 12°C. and 19.1°C.

The occurrence of Trachelomonas hispida (graph 2, chart 4) in winter is discontinuous and the number represented is also small. The individuals increased from early spring to the middle part of June, and a well-defined maximum was found in autumn. On this account, T. hispida seems to be favored by warm water. But this species disappeared in July, when the temperature of water was the highest.

In contrast with the Sarcodina, there are many stenothermal species among the Mastigophora. The temperature relations of Phacus pleuronectes, P. longicaudatus, and P. anacoelus are quite limited and similar among themselves. The thermal ranges for them were 12°C. to 25.9°C. for P. pleuronectes (graph 3, chart 4), 11.4°C. to 25.9°C. for P. longicaudatus, and 12°C. to 24.3°C. for P. anacoelus. It is worth while to note that P. longicaudatus appeared as early as the week of March 14th to 20th, accompanied by unusually high temperatures reaching to 13°C., but it immediately disappeared for three successive weeks when the temperature fell below 10°C., and reappeared when the temperature rose to 11.4°C. Other thermophilic flagellates are Trachelomonas armatus, T. urceolata, and Leptocinclis ovum.

Among the psychrophilic flagellates, Dinobryon sertularia, Synura uvella, Gonium pectorale, and Chlamydomonas pulviculus need special mention. Synura uvella (graph 3, chart
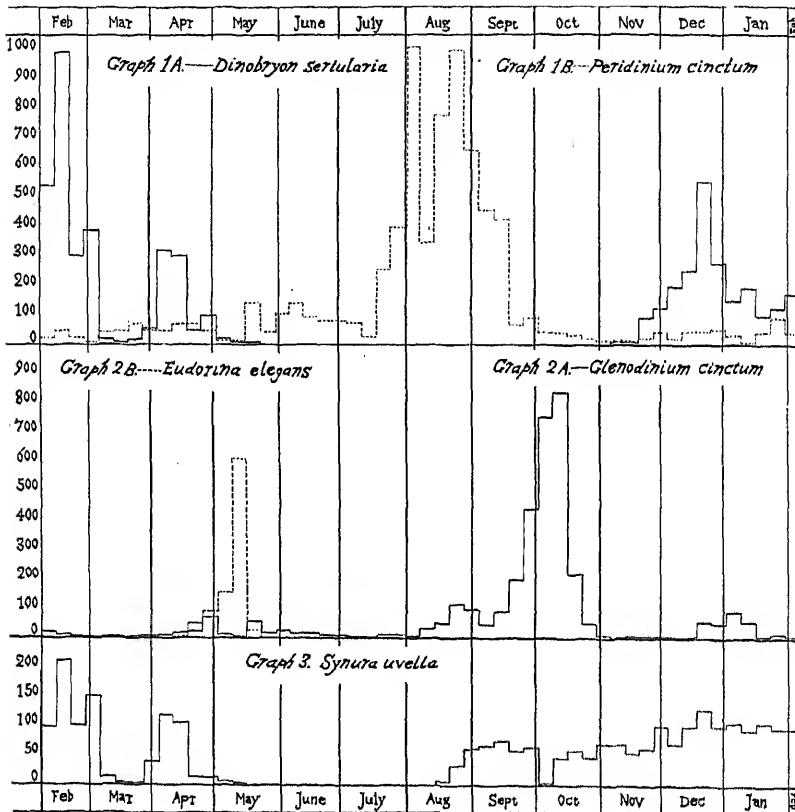
Chart 5 Showing the seasonal distribution of certain colonial and dinoflagellates. Graph 1A, Dinobryon sertularia. Graph 1B, Peridinium cinctum. Graph 2A, Glenodinium cinctum. Graph 2B, Eudorina elegans. Graph 3, Synura uvella. Ordinates indicate the number of animals per cubic centimeter.

5) and Gonium pectorale, with their largest population in cold water, were not observed at all when the temperature was above 21°C. The occurrence of Dinobryon sertularia (graph 1A, chart 5) was limited to temperatures below 18°C., with its maximum abundance at a temperature of 4.1°C.

Within a rather narrow thermal range (1.1°C. to 13.2°C.), Chlamydomonas pulviculus represents an extreme case of psychrophilic distribution.

Uroglena americana and Eudorina elegans (graph 2B, chart 5) presented a short period of occurrence, but with enormous numbers in the spring, when the temperature was neither high nor low, but changing from lower to higher. These flagellates cannot be designated as either thermophylic or psychrophylic, but they are definitely stenothermic.

Infusoria. Strombilidium gyrans, Frontonia acuminata, Coleps hirtus, Chilodon cucullulus, Dileptus gigas, Lionotus fasciola, Cinetochilum margaritaceum, Cyclidium glaucoma, Paramecium trichium, Stentor polymorphus, Uroleptus piscis, Stylonychia mytilis, S. pustulata, Aspidisca costata, and Campanella sp.? are all eurythermic ciliates, since they were found frequently or infrequently in all seasons under wide variations in temperature. In most of the species the largest population is, however, limited to warm days. According to their maximum abundance, the favorable temperature is about 22°C. for Frontonia acuminata (graph 4A, chart 6) and Campanella sp.? (graph 3, chart 7), 19°C. for Coleps hirtus (graph 1, chart 6), 18°C. for Strombilidium gyrans, Cyclidium glaucoma (graph 4B, chart 6), Paramecium trichium, and Stylonychia mytilis. In contrast with these, Aspidisca costata (graph 2, chart 7) and Stylonychia pustulata have their respective maxima at temperatures of 7.2°C. and 4°C.

Judging from their constant occurrence only in warm parts of the year, Prorodon griseus, Holophrya kessleria, Askenasia elegans, Arachnidium bipartitum, Urocentrum turbo, Spirostomum teres, Halteria grandinella, and Euplotes patella are thermophilic species. In comparing the graphs representing some of these species with the graphs for temperature, one will notice the different degrees of adaptation toward higher temperatures as exemplified by these species. Halteria grandinella (graph 2, chart 6) seems to be favored by the highest temperature, while it may also live at a thermal point

as low as 7°C. The thermal range becomes narrower in the case of Euplotes patella (graph 1B, chart 7). Urocentrum turbo (graph 6B, chart 6) lives within the thermal range of 14.5°C. to 22.4°C. Arachnidium bipartitum (graph 5B, chart 6) has never been observed in samples below 15°C., while Holophrya kessleria (graph 3B, chart 6) only occurs in the water having a temperature above 20°C.

There are a few species of psychrophilic ciliates, including Holophrya gargamella, Paramecium bursaria, Strombidium viridis, S. claparèdii, Urostyla grandis, Oxytricha pellionella, and Pleurotricha lanceolata. Paramecium bursaria (graph 5A, chart 6) is usually found in great numbers during the winter, but it may also appear when the temperature is as high as 21°C. The occurrence of Holophrya gargamella (graph 3A, chart 6) is confined to temperatures between 1.1°C. and 14.6°C., and in several instances, it was observed to die off very quickly at room temperature (23°C. to 25°C.) in the laboratory. Here, however, is a paradoxical relation in that, with a general tendency of distributing toward the lower temperatures, H. gargamella showed maximum numbers in the week of March 14th to 20th, when there was an unusual increase of water temperature to 13.2°C. Strombidium viridis disappeared above 18°C., while S. claparèdii (graph 6A, chart 6) and Oxytricha pellionella (graph 1A, chart 7) were only found below 15.6°C.

The thermal ranges for Teuthophrys trisulca (graph 7, chart 6) and Cyclotrichium vernalis are extremely limited, since they only occurred for a few weeks in the later spring. Like Uroglena americana and Eudorina elegans in the Mastigophora, they are definitely stenothermic.

Chart 6 Showing the seasonal distribution of certain holotrichous and oligo-trichous ciliates. Graph 1, Coleps hirtus. Graph 2, Halteria grandinella. Graph 3A, Holophrya gargamella. Graph 3B, Holophrya kessleria. Graph 4A, Frontonia acuminata. Graph 4B, Cyclidium glaucoma. Graph 5A, Paramecium bursaria. Graph 5B, Arachnidium bipartitum. Graph 6A, Strombidium claparèdii. Graph 6B, Urocentrum turbo. Graph 7, Teuthophrys trisulca. Ordinates indicate the number of animals per cubic centimeter.
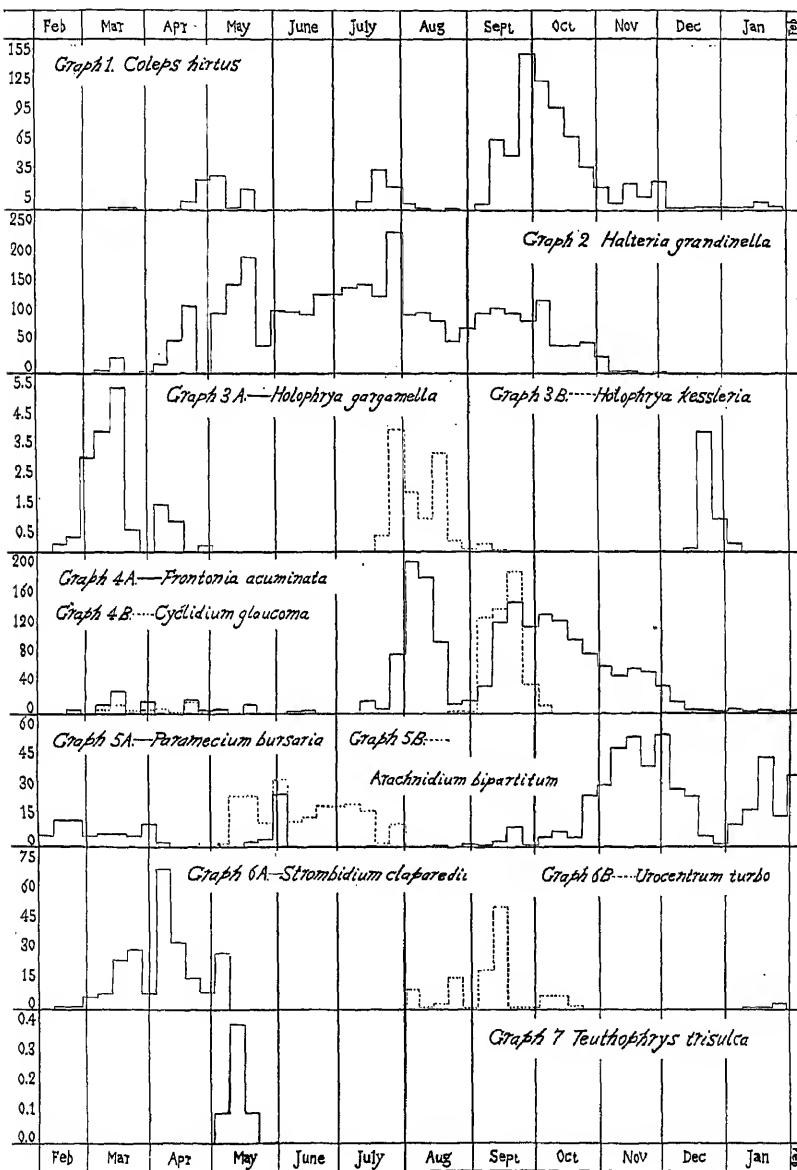
Chart 6

*E. Correlation between oxygen and the seasonal distribution
of protozoa*

Sarcodina. Most of the species of Sarcodina tolerate a
wide range of oxygen content, but, according to their seasonal
distribution, they are more numerous in the lower ranges of
oxygen content. A major maximum of abundance for Difflugia
lobostoma occurred (week of August 1st to 7th) when the
water contained only 3.05 cc. per liter of oxygen, and the
amount of this dissolved gas was still less (2.3 cc. per liter)
when a minor maximum of this Difflugia was recorded in the
week of July 11th to 17th (graph 1, chart 3, and graph 2,
chart 1). Actinosphaerium eichhornii appeared within the
range of 2.77 cc. to 14.38 cc. per liter, but its maximum oc-
curred when the water contained 6.36 cc. per liter. Amoeba
polypodia (graph 2, chart 3) and Nebela dentistoma (graph
3, chart 3) appear to represent two divergent types of adapta-
tion toward limitation by oxygen content. Amoeba poly-
podia was found only when the water contained from 2.3 cc.
to 7.8 cc. per liter of oxygen. On the other hand, Nebela
dentistoma was observed only when the range of oxygen
content was 6.2 cc. to 15.3 cc. per liter.

Mastigophora. Euglena viridis, Trachelomonas hispida,
Glenodinium cinctum, and Peridinium cinctum can tolerate a
great variation in oxygen content of the water from the low-
est to the highest. Their respective numerical maxima are,
however, found within the oxygen range of 3 cc. to 9 cc.
per liter.

Other species of Euglena and Trachelomonas, as well as
those of Phacus, appeared in water having a comparatively
low oxygen content. The same thing is true for Leptocinclis
ovum and Peranema trichophorum.

In contrast with those mentioned above, some of the
colonial flagellates were habitually found in the pond when
the oxygen content was high. Dinobryon sertularia (graph
1A, chart 5) had just appeared (week of November 7th to
13th) when the dissolved oxygen in water was above 6.4 cc.
per liter, and a major maximum occurred when the oxygen

content was 11.5 cc. per liter with a minor one at 15.3 cc. per liter. Although Synura uvella has been observed when the oxygen content was as low as 4.6 cc. per liter, its two maxima were recorded at the same time (February 14th to 20th and December 19th to 25th) as those of Dinobryon sertularia when the oxygen ranges were very high. With a maximum abundance when the oxygen content was 12.86 cc. per liter, Gonium pectorale was not collected in water having less than 6.3 cc. of oxygen per liter. A non-colonial flagellate, Chlamydomonas pulviculus, appears to be an extreme case of limitation to high oxygen content, having a range from 9.08 cc. to 15.3 cc. per liter.

Narrow ranges of oxygen content of 6.4 cc. to 8.64 cc. per liter for Uroglena americana and 6.4 cc. to 9.08 cc. per liter for Eudorina elegans (graph 2B, chart 5) were recorded.

Infusoria. The species of Infusoria having the widest oxygen range are Coleps hirtus, Dileptus gigas, Lionotus fasciola, Frontonia acuminata, Cinetochilum margaritaceum, Paramecium trichium, Stentor polymorphus, Strombilidium gyrans, Halteria grandinella, Stylonychia pustulata, Aspidisca costata, and Campanella sp.? The maximum populations for Halteria grandinella (graph 2, chart 6) and Frontonia acuminata (graph 4A, chart 6) were recorded when the oxygen content was less than 4 cc. per liter. Aspidisca costata (graph 2, chart 7) and Stylonychia pustulata were most numerous at the high oxygen content of 12.2 cc. and 11.82 cc. per liter, respectively. The oxygen readings recorded for the maxima in other ciliates mentioned above are neither very low nor very high.

Holophrya gargamella, Strombidium viridis, S. claparèdii, Paramecium bursaria, Oxytricha pellionella, and Pleurotricha lanceolata all appeared in water having a high oxygen content.

Holophrya gargamella appeared only when the oxygen content was above 8.64 cc. per liter (graph 3A, chart 6, and graph 2, chart 1). Like its relation to temperature, this species shows another paradoxical correlation. A minor

maximum was recorded when the oxygen content was 15.3 cc. per liter—the highest. The major maximum occurred when there was a sudden diminution of oxygen from 11.3 cc. to 9.48 cc. per liter. This major maximum was followed immediately by a great decrease in numbers, which suggests a lag in the reaction of these animals to the changes in oxygen content. The oxygen range is above 7.0 cc. per liter for Strombidium claparèdii (graph 6A, chart 6, and graph 2, chart 1) and above 6.4 cc. per liter for Strombidium viridis. The former had a maximum abundance when the oxygen content was 11.0 cc. per liter and the latter when it was 12.8 cc. per liter. Notwithstanding the fact that Paramecium bursaria tended to thrive best when the oxygen concentration was high, it was also found in water with the oxygen content as low as 4.65 cc. per liter, and its numbers were also decreased to a considerable degree when the highest oxygen content was recorded. Oxytricha pellionella was not observed when the water contained less than 7.0 cc. per liter of oxygen (graph 1A, chart 7, and graph 2, chart 1), and it reached its maximum numbers when the oxygen content was a little over 14 cc. per liter. The occurrence of Pleurotricha lanceolata was confined within the range of 11.8 cc. to 14.9 cc. per liter.

The ciliates which were habitually found in water of low oxygen content were Actinobolus radians, Prorodon griseus, Holophrya kessleria, Askenasia elegans, Urocentrum turbo, Spirostomum teres, and Stichotricha secunda. Prorodon griseus was found in water with oxygen content as high as 11.3 cc. per liter, but its maximum abundance was recorded when the water contained only 2.77 cc. per liter. The same thing is true for Askenasia elegans, since its oxygen range is from 2.3 cc. to 10.7 cc. per liter. The optimal limitations of low oxygen content in cubic centimeters per liter for other species are summarized as follows: Actinobolus radians, 2.77 cc. to 6.7 cc.; Holophrya kessleria, 2.77 cc. to 6.7 cc. (graph 3B, chart 6, and graph 2, chart 1); Spirostomum teres, 2.77 cc. to 7.8 cc.; Urocentrum turbo, 3.05 cc. to 7.8 cc. (graph 6B, chart 6, and graph 2, chart 1), and Stichotricha secunda, 3.82 cc. to 7.5 cc.

Correlated with a short period of occurrence, certain ciliates presented a very limited oxygen range. Thus the oxygen range was only 6.4 cc. to 8.64 cc. per liter for Cyclotrichium vernalis and 6.4 cc. to 7.0 cc. per liter for Teuthophrys trisulca (graph 7, chart 6, and graph 2, chart 1).
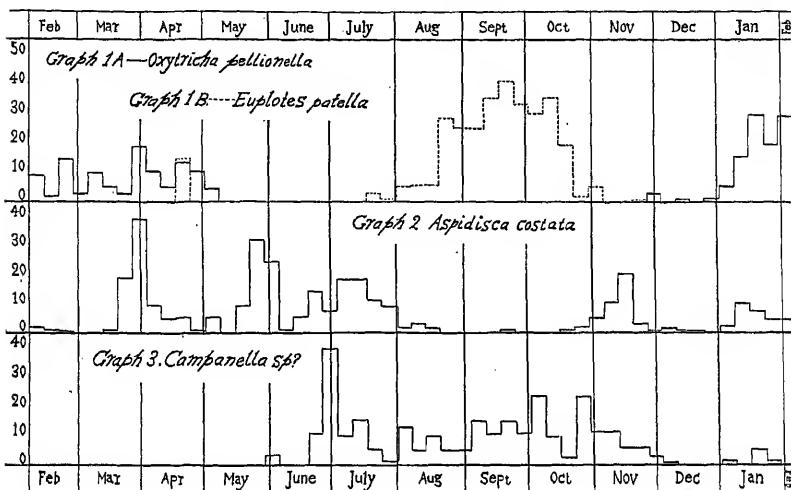
Chart 7 Showing the seasonal distribution of certain hypotrichous and peritrichous ciliates. Graph 1A, Oxytricha pellionella. Graph 1B, Euplotes patella. Graph 2, Aspidisca costata. Graph 3, Campanella sp.? Ordinates indicate the number of animals per cubic centimeter.

F. Correlation between hydrogen-ion concentration and the seasonal distribution of protozoa

Sarcodina. As we have stated above, the pH value for the pond water studied is fairly constant throughout the year, exhibiting a limited range from 6.2 to 7.05. Actinosphaerium eichhornii, Amoeba discoides, Dactylosphaerium radiosum, Difflugia lobostoma, and D. corona have been observed within these extremes of the pH range.

In the case of Amoeba polypodia and Arcella discoides, the optimal pH range appears to be rather narrow, from 6.6 to 7.05 (graph 2, chart 3, and graph 3, chart 1) and from 6.52 to 7.0, respectively. Both of these species disappeared to-

gether from October 31st onward after a sudden decrease of pH from 6.6 to 6.2 had occurred.

Nebela dentistoma, on the other hand, seems to be favored by the lower pH of 6.2 to 6.7 (graph 3, chart 3, and graph 3, chart 1).

Mastigophora. Peridinium cinctum and Glenodinium cinctum were found within the entire range of the observed pH, but their populations were very small when the pH was less than 6.4 (graph 1B, 2A, chart 5, and graph 3, chart 1). The limitation of pH range for Euglena viridis was 6.35 to 7.05 (graph 1, chart 4, and graph 3, chart 1) and that for Phacus pleuronectes was 6.5 to 7.05 (graph 3, chart 4, and graph 3, chart 1). Like Amoeba polypodia and Arcella discoides, these two flagellates also disappeared when the pH dropped to 6.2. A trace of Trachelomonas hispida was found in the first week of the lowest pH, but it likewise disappeared in the second week when the pH was unchanged (graph 2, chart 4, and graph 3, chart 1). Other flagellates, except three species of the colonial forms to be considered below, were never found when the pH was as low as 6.2.

Synura uvella, Gonium pectorale, and Dinobryon sertularia have the same pH ranges, namely, from 6.2 to 6.91. The numbers of Synura and Gonium were not decreased (nor increased) in the water showing pH of 6.2, while Dinobryon, after a long absence during the summer and early autumn, reappeared in the second week of the period of lowest pH.

The pH range appeared to be extremely limited (6.75 to 6.9) in the case of Uroglena americana and Eudorina elegans.

Infusoria. The ciliates observed to occur within the entire pH range of 6.2 to 7.05 are the following: Coleps hirtus, Askenasia elegans, Trachelius subtilis, Dileptus gigas, Lionotus fasciola, Frontonia acuminata, Cinetochilum margaritaceum, Halteria grandinella, Strombilidium gyrans, Euplotes patella, Aspidisca costata, Vorticella campanula, V. nebulifera, and Campanella sp.?

The pH range seems to be limited for certain ciliates. Examples are: Strombidium claparèdii and S. viridis, 6.4 to

6.96; Holophrya gargamella, 6.4 to 6.9; Prorodon griseus and Arachnidium bipartitum, 6.52 to 7.05; Urocentrum turbo, 6.6 to 6.88; Holophrya kessleria, 6.65 to 7.05, and Teuthophrys trisulca and Cyclotrichium vernalis, 6.75 to 6.91.

Chilodon cucullulus, Frontonia leucas, Lembadion bullinum, Craspedonotus vermicularis, and Spirostomum teres were not recorded when the pH of the water was as low as 6.2.

Stentor polymorphus seems to be favored by lower pH, since its maximum of abundance was found when the pH of water was just 6.2. Perhaps it is also true for other species of Stentor. Thus, according to their maxima in occurrence, the optimal pH is 6.4 for S. roseus, 6.45 for S. barrettii, and 6.6 for S. coeruleus.

G. Correlation between volatile acids and the seasonal distribution of protozoa

In presenting the data on the volatile acids in relation to the seasonal distribution of protozoa, the numbers on the scale for graph 4, chart 1, will be utilized to designate the relative amount of volatile acids. These numbers, indicated everywhere in this paper as difference of pH or pH difference, are, therefore, not directly related to the ordinary pH value. It will be understood, of course, that the larger the difference in pH after boiling, the larger would be the amount of volatile acids which were originally present in the water.

Sarcodina. Actinosphaerium eichhornii, Raphidiophrys elegans, and Difflugia lobostoma were found within the widest range of the concentration of volatile acids in the pond water. However, they became numerous only in the lower concentration of such acids. At the time of their seasonal maxima, the difference between the two readings of pH is 0.64 for Actinosphaerium eichhornii, 0.65 for Difflugia lobostoma (graph 1, chart 3, and graph 4, chart 1), and 0.7 for Raphidiophrys elegans.

The occurrence of Amoeba polypodia was limited to pH differences between 0.4 and 0.83 (graph 2, chart 3, and graph 4, chart 1). Other Sarcodina were usually not found in abun-

dance when the surface water contained a great amount of volatile acids.

Nebela dentistoma is the only form that frequently occurred in a higher range of the pH difference (0.64 to 2.0).

Mastigophora. The flagellates which occurred within the widest limits of the relative amount of volatile acids are Euglena viridis, Glenodinium cinctum, and Peridinium cinctum. Trachelomonas hispida also has a wide range in regard to such acids, but it never occurred at the upper and lower extremes.

Euglena deses, Trachelomonas armatus, Phacus anacoelus, Phacus pleuronectes, and Peranema trichophorum were more numerous in water having a low content of volatile acids. Thus optimal volatile-acid content is indicated by pH differences of 0.5 for Euglena deses, 0.7 for Trachelomonas armatus and Peranema trichophorum, and 0.45 for Phacus anacoelus and P. pleuronectes (graph 3, chart 4, and graph 4, chart 1).

Those flagellates which seemed to be favored by a high content of volatile acids are Synura uvella, Dinobryon sertularia, Chlamydomonas pulviculus, and Gonium pectorale. The ranges of pH difference recorded were 0.68 to 2.0 for Synura uvella (graph 3, chart 5, and graph 4, chart 1) and Gonium pectorale, 0.8 to 2.0 for Dinobryon sertularia (graph 1A, chart 5, and graph 4, chart 1), and 1.2 to 2.0 for Chlamydomonas pulviculus.

Uroglena americana and Eudorina elegans were found where a moderate amount of volatile acids was present.

Infusoria. Coleps hirtus, Dileptus gigas, Lionotus fasciola, Frontonia acuminata, Cinetochilum margaritaceum, Paramecium trichium, Stentor polymorphus, Strombilidium gyrans, Halteria grandinella, Stylonychia pustulata, Aspidisca costata, and Campanella sp.? have been observed to occur within a wide range of concentration of volatile acids dissolved in the water.

Except in the case of Stylonychia pustulata and Aspidisca costata, the individuals of the above-mentioned species were

more numerous when the concentration of volatile acids was comparatively low.

Certain ciliates were found only when the concentration of volatile acids was low. The seasonal maxima in numbers for Prorodon griseus and Holophrya kessleria were recorded when the water had the lowest concentration of volatile acids. The range of the difference of pH is 0.4 to 0.8 for Spirostomum teres, 0.4 to 0.82 for Actinobolus radians, 0.4 to 1.4 for Askenasia elegans, 0.65 to 0.74 for Urocentrum turbo (graph 6B, chart 6, and graph 4, chart 1), and 0.8 to 1.1 for Teuthophrys trisulca (graph 7, chart 6, and graph 4, chart 1) and Cyclotrichium vernalis.

Ciliates usually found in water having a high concentration of volatile acids are Holophrya gargamella, Oxytricha pellionella, Strombidium claparèdii, S. viridis, and Paramecium bursaria. Holophrya gargamella, Oxytricha pellionella, and Strombidium claparèdii have the same range of pH difference (1.0 to 2.0), while the range is 0.8 to 2.0 for Strombidium viridis and 0.64 to 2.0 for Paramecium bursaria (graph 5A, chart 6, and graph 4, chart 1).

H. Other physical factors of the environment influencing the seasonal distribution of protozoa

Sunlight versus cloudiness. Since my data on the weather conditions were made only at the time of collection and since there might be a lag in the effect of sunlight on the occurrence of the protozoa, the monthly records, composed of daily estimations obtained from the U. S. Weather Bureau in Philadelphia, were utilized. These monthly records are entered in table 1.

By a glance at the table one will notice that there were more sunny days in September (fourteen) and October (eighteen), 1927, than in any other months of the year covered by this record. It is interesting to note that the seasonal maxima of most of the species of protozoa were recorded during these two months. Very few species reached their maxima in August when there were only three clear days in the month.

The numbers of Actinosphaerium eichhornii, Raphidio-phrys elegans, and Clathrulina elegans were always small on cloudy days, and they also increased in number, particularly in September, when a great amount of sunlight was available. Although not recognized as a typical heliotropic species, Difflugia lobostoma, after the seasonal maximum recorded during the first week of August, suddenly decreased in number with an accompanying decrease in sunshine in that month. During the first part of September, when the sunlight was sufficient, the individuals of D. lobostoma tended to increase again, and it remained numerous throughout most of October.

TABLE 1

Monthly records on the conditions of sunshine obtained from the U. S. Weather Bureau in Philadelphia (data for February 1, 1927, to January 31, 1928, inclusive)

	NUMBER OF DAYS, 1927											1928
	February	March	April	May	June	July	August	September	October	November	December	January
Clear	5	10	12	3	7	7	3	14	18	5	9	13
Partly cloudy	11	8	4	14	12	13	10	9	3	9	6	9
Cloudy	12	13	14	14	11	11	18	7	10	16	16	9

Of the Mastigophora, species of Euglena and Phacus are particularly favored by sunlight. Their numbers usually decreased on cloudy days as compared to sunny days. The population of Euglena viridis was constantly greater in March and April, when there were relatively more clear days, and gradually decreased during May, when the duration of sunlight was less. From fairly large numbers in the latter part of July, the population of Euglena viridis decreased in August, when the sunlight was particularly deficient, increased again at the end of September, and reached a minor maximum in the early part of October when the sunlight was favorable. Phacus pleuronectes showed the same reaction to sunlight, since the numbers were rather small in the relatively dark months of May and August.

Ciliates which appear to be favored by sunlight are: Coleps hirtus, Holophrya gargamella, Chilodon cucullulus, Teuthophrys trisulca, Cyclotrichium vernalis, Strombidium viridis, and S. claparèdii. With a seasonal maximum in the week between September and October, when the sunlight was extensive, Coleps hirtus might be considered as a heliotropic species. A noticeable depletion in population of this species was also recorded in August, when the cloudiness was most pronounced. The numbers of the other ciliates, mentioned above, tended to decrease on cloudy days as noted on my own data sheets. Teuthophrys trisulca and Cyclotrichium vernalis are very susceptible to cloudiness, since they were very scarce when the sun was not shining. The population of these two species was extremely small during the excessively cloudy month of May, 1927, as compared with several preceding years according to records kept by Dr. D. H. Wenrich.

Effect of rain. The effect of rain on the distribution of protozoa at the surface of the pond is rather a direct one. Traces of Actinosphaerium eichhornii may be found on cloudy days, but were never recorded on rainy days. Euglena viridis disappeared during rain, while, in certain cases, there were more individuals of Peridinium cinctum on rainy days than on clear days. Ciliates which were more likely to disappear during rain are Askenasia elegans, Cinetochilum margaritaceum, Lembadion bullinum, Paramecium trichium, Stentor polymorphus, Uroleptus piscis, Oxytricha pellionella, O. platystoma, and Tachysoma parvistyla.

It is of interest to note that, although the numbers of protozoa might diminish greatly when it rained, the numbers of individuals of various species tended to increase again just after the rain. On August 18th and November 28th (cloudy days), the numbers of individuals were much larger than those found on preceding or succeeding days while there were very heavy rains during the night preceding the collections.

Winter conditions. In winter the pond was often covered with ice, and a chisel was used to make a hole for taking the

samples of water. The temperatures of the water just below the ice were always above 1.0°C. Eurythermic as well as psychrophilic species of protozoa were commonly present in the surface water (below the ice) when the pond was frozen. It may be said, therefore, that the diminution of protozoan population in winter was primarily due to the lower temperature, but not to the frozen condition.

Pieces of ice were also brought into the laboratory and allowed to melt in a sterile vessel. Examination of the water from the melted ice failed to reveal any protozoa. Unlike certain algae, therefore, protozoa seem not to become frozen in the ice.

DISCUSSION

While some papers have been published on the general ecology of protozoa, information concerning the habitats of individual species is frequently found in taxonomic studies. Those of Leidy ('79), Kent ('80), Stokes ('88), Roux ('01), Penard ('02, '22), Fauré-Fremiet ('24), and Kahl ('26, '27) are examples.

Studies of the various physical, chemical, and biological conditions of laboratory cultures of protozoa have been reported by Peters ('07), Woodruff ('10, '12), Fine ('12), Jacobs ('12), and Bodine ('21).

Studies of protozoa in relation to more natural environments have been made, among others, by Lauterborn ('01), Juday ('08, '19), Kolkwitz and Marsson ('09), Hausmann ('17), Riggenbach ('22), Rylov ('23), Crozier ('23), and Noland ('25).

Hausmann grouped the fresh-water protozoa into five environmental types in regard to special habitats without measuring any of the physical or chemical conditions of the different environments. Crozier found that, in the surface film of the filter bed of a sewage-disposal plant, the number of kinds of ciliates or rhizopods is directly correlated with the abundance of individuals, in contrast with the inverse correlation often found under more natural conditions, and suggested that study of such relationships might afford a basis

for comparing the 'selective potentials' of different environments. Lauterborn applied the term 'sapropelic fauna' to those protozoa which may live in putrefying detritus at the bottom of pools where hydrogen sulphide seemed very abundant. Juday listed certain protozoa which appeared to exist without free oxygen at the bottom of Lake Mendota, and later he also published an account on an anaerobic ciliate. Kolkwitz and Marsson classified the protozoa into three groups, namely, 'polysaprobe,' 'mesosaprobe,' and 'oligosaprobe,' according to the progress of self-purification in polluted water from which the animals were collected and observed. Rylov studied the correlations between seasonal conditions in regard to the dissolved oxygen and hydrogen sulphide and the vertical distribution of Loxodes rostrum. Riggenbach divided the heliozoans and ciliates into three groups, according to their distribution toward a higher oxygen content on the one hand and higher content of carbon dioxide, hydrogen sulphide, and ammonia on the other. Noland found that certain ciliates are favored by warm water, while some were found chiefly in cold water; ciliates living on algae occurred in water having a high oxygen and low carbon-dioxide content, while those feeding on bacteria were found where the oxygen content was low and carbon dioxide was much more abundant. Both Riggenbach and Noland seem to have concluded that the nature and amount of available food present in the water exert a direct influence on distribution of the protozoa.

Abundance and diversity in the seasonal distribution of protozoa

As pointed out by Crozier ('23), in a natural environment, the number of kinds of planktonic organisms as well as other forms is often inversely correlated with the number of individuals at any seasonal period. Such correlations among the major groups of protozoa have been briefly mentioned in the preceding pages (pp. 445–447).

If comparison is made among certain genera, the inversed correlation between abundance and diversity is clearly illustrated. Dinobryon sertularia, Peridinium cinctum, and Glenodinium cinctum are the only species in their respective genera found in the pond, yet the maximum of each greatly surpasses the combined maxima of six species of Euglena, four species of Phacus, and three species of Trachelomonas. There were large numbers of individuals of Halteria grandinella and Strombilidium gyrans, which were also the only species in these genera recorded. Coleps and Frontonia are each represented by two species, and these ciliates were frequently found in great abundance. A number of species of Paramecium, Stentor, Oxytricha, and Vorticella has been observed, but the numbers of individuals were small (except Paramecium bursaria) as compared with those of Halteria, Strombilidium, Coleps, and Frontonia.

It has been long considered that allied species of the same genus living in the same locality may require the same sorts of environment, and therefore competition arises among them in maintaining their existence. It may be supposed that species of Euglena, Phacus, Paramecium, Stentor, etc., had to meet with such competition in this particular pond which provided a natural check to their increase in numbers. On the other hand, the very large numbers of Dinobryon sertularia, Peridinium cinctum, Halteria grandinella, Strombilidium gyrans, etc., might be expected, since they had no competitors in closely related species.

Distribution of bottom dwellers and thigmotropic species

The Sarcodina, except certain Heliozoa, are considered to be principally bottom dwellers, and, therefore, the records from the surface collections probably are quite inadequate to determine the seasonal distribution of the members of this group. Just what factors are responsible for bringing them to the surface have not been determined, though convection currents or vertical currents, caused by wind action, and reactions to food conditions may be mentioned as possible agents.

The majority of peritrichous ciliates, such as Vorticella, Opercularia, Zoothamnium, Vaginicola, Rhabdostyla, and Trichodina and adult Suctoria, are found to be attached to foreign objects by means of stalks, pedicels, disks, or other attachments. They may or may not be associated with other organisms, but ·the occurrence of certain associated infusorians is directly correlated with the incidental distribution of their hosts in the surface water, and the records made on their seasonal distribution are not comparable to those of the surface roamers.

It should be mentioned, however, that the data for the distribution of Campanella sp.? were made on the free-swimming zoids, the distribution of which seemed to parallel the seasonal variations in the environment.

carbonates of calcium and magnesium

pH

oxygen content ←——— temperature ———→ concentration of volatile acids
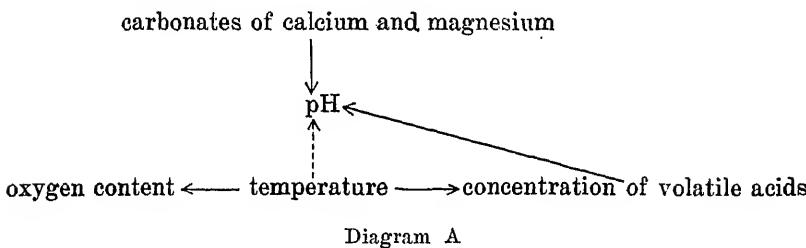
Diagram A

Interrelations among the physicochemical factors of the environment

The data showing correlations in the seasonal variations among the physical and chemical factors of the environment have already been presented (pp. 443 and 445). Temperature is probably the main factor in influencing the chemical changes, particularly in the surface water from which the measurements were derived. As pointed out in Whipple ('27), the gases may be conveyed from the air to the water by three main processes: solution, convection currents, and diffusion. The rate of each of these processes is directly influenced by the temperature. As illustrated by diagram A, temperature exerts a direct influence on both oxygen content and concentration of volatile acids and an indirect influence

on pH value (indicated by discontinuous arrow) which is directly effected by volatile acids and checked, probably, by the presence of carbonates of calcium and magnesium.

Correlation between temperature and the distribution of protozoa

Most of the species of protozoa seemed much more susceptible to thermal changes than to the fluctuations of other environmental factors. Thermophylic or psychrophylic species appeared to have definite ranges of optimal temperature. Among the thermophylic forms, Halteria grandinella, Spirostomum teres, and Urocentrum turbo were also reported by Noland ('25) in the high-temperature group. Lauterborn included Dinobryon sertularia and Synura uvella in his "Winter fauna," and Noland recognized Oxytricha pellionella as favored by low temperature. As determined by the present study, these three species together with a number of others are typical psychrophylic protozoa.

It is possible that the chemical processes associated with metabolic activities of the protozoan cells can be accelerated by increasing temperatures in accordance with van't Hoff's law. The increase of the rate of metabolism may lead to a rapid process of reproduction (assuming an adequate food supply), and this has been indicated by the fact that the greatest numbers of eurythermic as well as thermophylic species were recorded on warm days, and even the maxima of psychrophylic forms never occurred at the lowest temperature. It is of interest to note that binary fission of Halteria grandinella and conjugation of Frontonia acuminata were usually observed in the fresh sample when the temperature of the water was high.

The relations between protozoa and the chemical environment

Oxygen is one of the most important requirements for carrying on the metabolic activities of all animals, and the great abundance of certain free-swimming protozoa in this pond is probably correlated with the fact that the surface

water is well oxygenated in most parts of the year. Some protozoa, however, appear to be favored by a considerable diminution of oxygen in the medium in which they live. In this connection it may be noted that those species listed by Kolkwitz and Marsson as 'polysaprobien' and species like Microthorax sulcatus, Caenomorpha medusula, and Metopus sigmoides, recorded by Riggenbach ('22) and Noland ('25) as living in water having the lowest oxygen content and highest carbon-dioxide, or hydrogen-sulphide, or ammonia content, were never observed in the surface water of this pond, where a considerable amount of free oxygen was always available. Spirostomum ambiguum, recognized by Pütter ('04) as a facultative anaerobe, and Plagiopyla nasuta, reported by Riggenbach as living in a high concentration of carbon dioxide, hydrogen sulphide, etc., appeared in the surface only once in the year in extremely small numbers.

All the colonial flagellates are chlorophyll-bearing protozoans and were found in great abundance during the winter when the water contained much more oxygen and volatile acids, especially carbonic acid. The larger amount of carbonic acid could be utilized by the chlorophyll in photosynthesis which, in turn, would tend to produce an excessive amount of oxygen in the water. The interrelations between the chemical surroundings and Paramecium bursaria are very striking. The Zoochlorellae or symbionts must have sufficient quantity of carbon dioxide for their photosynthetic processes, while P. bursaria itself seemed to be favored by a higher concentration of oxygen.

Cowles and Schwitalla ('23) pointed out that the constant pH value of 6.5 is most favorable for the development of Euglena viridis, after they had studied the hydrogen-ion concentration of a stream and a series of pools. The entire disappearance of Euglena viridis, together with some other flagellates as well as certain ciliates and Sarcodina in the pond here reported on, when the pH was 6.2, indicated that the hydrogen-ion concentration might be one of the limiting

factors for the distribution of these protozoa under natural
conditions.

Physical factors versus biological factors of the environment

Thus far in this discussion little attention has been given to
the food supply and enemies of the protozoa in the pond. The
protozoa, except certain chlorophyll-bearing flagellates, usu-
ally feed on bacteria, algae, rotifers, and other protozoa, and,
in turn, they may be also captured and eaten as food by dif-
ferent kinds of aquatic animals. Such biological relations in
nature are of equal importance to the physical factors of the
environment. The physical environment may have a direct
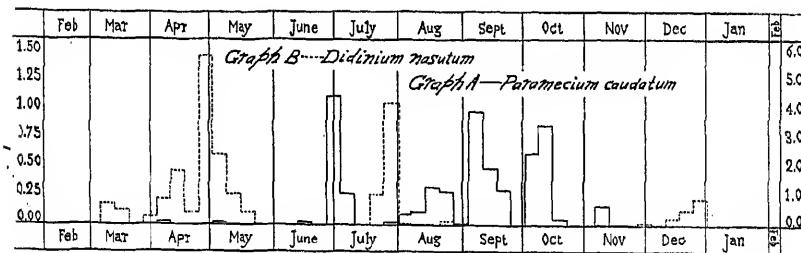
Chart 8 Showing the seasonal distribution of Paramecium caudatum (graph
A) and Didinium nasutum (graph B). Ordinates, at left, indicate the numbers
of P. caudatum per cubic centimeter. Ordinates, at right, indicate the numbers
of D. nasutum per cubic centimeter.

effect on the chemical processes of metabolism of the pro-
tozoan cells, while biological factors are intimately related to
nutrition and the problem of maintaining themselves in com-
petition with others.

Species of Paramecia have been considered as the most
favorable food for Didinium nasutum. The graphs on chart
8, however, indicate that there was no correlation between
the occurrence of Didinium nasutum and of Paramecium
caudatum. Not a single Paramecium caudatum was observed
in the week of April 25th to May 1st, when the maximum of
Didinium nasutum occurred, and, on the other hand, the
former became numerous only when the latter was totally

absent in the surface water. If the occurrence of P. aurelia and P. trichium were plotted in the same manner with Didinium nasutum, there would also be no correlation. This probably shows that Paramecium was able to escape from its enemy under natural condition, while Didinium nasutum had no opportunity to secure its supposedly favorite food.

As reported by Riggenbach ('22), Didinium nasutum also feeds on Halteria grandinella. In comparing the seasonal distribution of these two ciliates (graph B, chart 8, and graph 2, chart 6), one will notice that the correlation in their occurrence is well established in certain periods. The seasonal maximum of Halteria grandinella in the week of July 25th to 31st was accompanied by a great number of Didinium nasutum. In the week of April 25th to May 1st, when the maximum occurrence of Didinium nasutum was recorded, there was no trace of Halteria grandinella observed, while in preceding and succeeding weeks the individual numbers of the latter were over one hundred per cubic centimeter as correlated with fewer individuals of the former. In this particular week, it may be supposed that Halteria grandinella in the surface water either might all have been captured by the large numbers of Didinium nasutum or had migrated downward to the lower levels in avoiding the enemy. A minimum occurrence of Didinium nasutum from November 14th to December 4th was not associated with the presence of Halteria grandinella.

Adaptation of protozoa to the environment

The capacity of self-adjustment of protozoa appears to be much greater in the more cosmopolitan species than in less familiar ones. Species like Euglena viridis, Paramecium caudatum, Coleps hirtus, etc., are less hindered by the seasonal changes of the environment in a confined locality and also have a world-wide distribution. Teuthophrys trisulca has been recorded only in this pond and a lake in the Vosges Mountains of Europe and Cyclotrichium vernalis (Wenrich) is so far not known in other parts of the world. Both of

them seemed to be very susceptible to environmental changes, and hence their seasonal distribution was very limited.

Although changes in the physical environment do, undoubtedly, have a direct effect on the distribution of protozoa, the seasonal appearance or variations in numbers of certain species may indicate a rhythmical or cyclic series of events which are inherent in the organisms and not easily overcome by external conditions. Rhythmical changes in division rates, the cyclic occurrence of endomixis or conjugation under relatively constant laboratory conditions are well known, and recently Richards and Dawson ('27) found that the division rates of Paramecium aurelia, Blepharisma undulans, and Histrio complanatus in pedigreed isolation cultures exhibit a seasonal rhythm with a yearly maximum during July. Such results suggest that the seasonal changes in division rate and in other biological activity reflect a long process of adaptation between the protoplasmic organization and the changing environment, the resultant of these factors being the cyclic phenomena observed. However, in the further results reported by Richards and Dawson, as well as other instances cited by these authors, it is shown that the extent of seasonal variations diminishes with continued culture in the laboratory year after year, indicating that the relatively fixed cycle is still modifiable by an environment different from that to which they are normally subjected in nature.

SUMMARY

1. There were twenty-seven species of Sarcodina, thirty-one species of Mastigophora, and 109 species of Infusoria recorded in the surface water of the pond in the botanical gardens of the University of Pennsylvania from February, 1927, to February, 1928. The observed environmental factors in influencing the seasonal distribution of these protozoa were temperature, oxygen content, hydrogen-ion concentration, and the relative amount of dissolved acids.

2. Especially in the case of Mastigophora and Infusoria, the number of kinds is inversely correlated with the abundance of individuals in the seasonal distribution.

3. There were two seasonal maxima in the occurrence of Mastigophora as a whole. One, in winter, was chiefly due to the great abundance of Dinobryon sertularia and the other, in late summer, to the large numbers of Peridinium cinctum.

4. The quantitative determinations on the seasonal occurrence of the protozoa are significant only for the surface roamers, and not for bottom dwellers and thigmotropic species.

5. The amount of dissolved gases in the surface water is directly correlated with the temperature.

6. There were a great number of stenothermic species as well as eurythermic and more thermophilic species than psychrophilic. Higher temperatures probably accelerated the processes of metabolism which, in turn, may induce rapid reproduction, as indicated by the fact that the seasonal maxima for most of the species were recorded in warmer weather.

7. Colonial flagellates and zoochlorellae-bearing ciliates seemed to be favored by higher concentrations of oxygen with a simultaneous abundance of volatile acids (assuming a great amount of CO_2).

8. The hydrogen-ion concentration of the pond water is fairly constant throughout the year. It may be considered, however, as one of the limiting factors in protozoan distribution, since a number of species disappeared when the lowest pH, 6.2, was recorded.

9. Sunlight is one of the important factors in bringing certain heliotropic protozoa to the surface.

10. While the food supply may be considered as of equal importance with the physical environment, it has not been directly recorded. It is realized, however, that variations in seasonal abundance may depend on variations in food supply as well as on variations in the physical factors of the environment. In the case of Didinium nasutum, however, there was no correlation between its abundance and the numbers of Paramecium. It is possible that Didinium nasutum in this particular pond may also feed on Halteria grandinella, as reported by Riggenbach.

11. The seasonal maxima of most of the species were found during the months of September and October in the year 1927, when most of the observed physical environments seemed much more favorable for the protozoa than in other months.

12. The power of self-adjustment seems much greater in the more cosmopolitan species than in less familiar species, since the latter are probably more susceptible to the environmental changes, e.g., Teuthophrys trisulca recorded only in this pond and a lake in the Vosges Mountains of Europe. Thus the capacity of adaptation of various species is directly correlated with the extent and limitation of distribution.

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REACTIONS OF IMMATURE MONKEYS (MACACUS RHESUS) TO INJECTIONS OF OVARIAN HORMONE

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NINE HELIOTYPE PLATES (THIRTY-SIX FIGURES)

AUTHOR'S ABSTRACT

Series of injections of ovarian hormone have been made into normal and ovariectomized immature animals. Injections were made twice daily for twenty-two days. The total dose exceeded 1000 rat units per animal.

Effects noted in the living animals were the appearance of reddening and swelling of the 'sexual skin' and change of the cell content of the vaginal smear to the interval type of the mature animal. Measurements made at operation, before and after injections, indicated considerable enlargement of both the cervix and body of the uterus. The thymus glands of the injected animals weighed significantly less than those of the controls. Histologic study of the genital tract showed extreme thickening of the vaginal walls, considerable growth of the uterine epithelium and glands, hypertrophy of the muscle layers of the uterus, and advanced differentiation of the epithelium of the uterine tubes.

The ovaries of the injected normal animal were smaller and contained fewer primordial and medium-sized follicles than those of the controls. The presence of large numbers of atretic follicles, especially large flattened scars from former relatively well-developed follicles also suggests a harmful effect of this amount of ovarian hormone upon follicular development. Several stages of elimination of ova from polyovular follicles were also observed. There was marked growth in the ducts and an increase in the number of alveoli of the mammary glands.

INTRODUCTION

The effects of ovarian hormone upon immature animals have already been extensively studied in rats, mice, and rabbits (Fellner, '12, '13; Herrmann, '15; Allen and Doisy, '24; Frank, '22, '25; Brouha and Simonnet, '25, and Zondek and Aschheim, '26). The principal reaction is an acceleration of growth of the genital tract and mammary glands which develops these organs prematurely. The success of some of these tests has suggested the possibility of the clinical use of this hormone in the treatment of delayed attainment of puberty in human beings. Since the reproductive phenomena of monkey and man are so similar, the present work was undertaken to further investigate such possibilities. Especial

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emphasis has been placed upon the quantity of hormone, the time required for the reaction, and the degree of development resulting from the injections.

Another phase of this problem still awaits a definite answer, namely, the effect of injected ovarian hormone upon the ovaries of the immature animal. Apparent acceleration in growth of follicles in immature ovaries, resulting in precocious ovulation, has been reported. Conversely, in some cases an apparent inhibition of follicular growth and an increased atresia of follicles seem to follow injections of ovarian hormone. It is not definitely known whether the latter may be a transient effect from which the animal may later fully recover. Also, the dosage and reaction-time factors still require more exact investigation. Since the function of the other genital organs is so dependent upon properly functioning ovaries, a treatment for infantilism which inhibited ovarian development would not be rational, irrespective of the growth induced in the genital tract. ·Perhaps the hormone of the anterior lobe of the hypophysis may prove the proper therapy, as indicated by the recent work of Smith and Engle ('27) and of Zondek and Aschheim ('27). They have shown independently that daily implants of anterior lobe accelerate follicular development, which in turn accelerates the attainment of puberty. Negative results were obtained after removal of the ovaries, consequently the anterior lobe must work through the ovaries as intermediaries.

Since the writer, in conjunction with Dr. E. A. Doisy ('27 b), has recently reviewed the literature on this subject, reference will be made to this paper for a discussion of earlier contributions.

MATERIALS AND METHODS

Animals used

Three immature monkeys (Macacus rhesus) of approximately the same body weight were used: the first (LE) as a control, a second (SS) from which the ovaries had previously been removed, and the third (2E) normal, with ovaries intact, as test animals for injections.

Double ovariectomy was performed upon monkey SS on February 19, 1927. These ovaries served as controls before the series of injections were begun. At this operation the right uterine tube and left mammary gland were also removed for control specimens and measurements were taken of the uterus.

There was no exact record of age of these monkeys. At the time of operation SS weighed 2533 grams. The weights of the three animals on February 28, 1927, before injections were begun, were 2620, 2560, and 2660 grams (table 1). Their weights on March 23, 1927, at the end of injections, were 2705, 2686, and 2686 grams.

TABLE 1

Body weights in grams

DATE	ANIMALS			REMARKS
	LE, Control	SS, Spayed injected	2E, Normal injected	
2/19/27		2533		At ovariectomy
2/28/27	2620	2560	2660	Before injections
3/23/27	2705	2686	2686	After injections
Gain in weight	85	126	26	

These body weights may be compared with three other animals in our colony at the time of appearance of first menses (3130, 3170, and 4470 grams). This comparison and the fact that three other monkeys then at approximately the same body weight had not attained puberty on October 18, 1927, seven months later, at the weights of 3160, 3355, and 4120 grams, indicate that the experimental animals were quite immature.

None of the animals showed signs of reddening or swelling of the 'sexual skin' about the external genitalia which are due to the secretion of ovarian hormone during sexual maturity (Allen, '27 a).

Injections of hormone

Injections were begun into SS and 2E on March 2, 1927, eleven days after a double ovariectomy upon animal SS. Four different extracts were used as follows: 1) an oil solution of lipoid extract of liquor folliculi from pig ovaries (L.F. no. 17) containing more than 4 rat units of hormone per cc.; 2) an oil solution of lipoid extract of human placenta (P no. 28) containing more than 7 rat units per cc.; 3) an aqueous preparation (no. 093445-a) containing more than 25 rat units per cc., and, 4) a second aqueous preparation (no. 093486-a) containing 100 rat units per cc.[2]

Two injections were given daily over a period of twenty-two days. Normal animal 2E received a total of 1005 rat units, beginning with 8 rat units a day for the first ten days, increasing to 25 rat units a day from the eleventh to thirteenth days, and to 100 rat units a day for the last eight days. Ovariectomized animal SS received a total of 1065 rat units, beginning with 14 rat units daily for the first ten days and finishing the experiment with the same dosage as given to 2E. The maximum dose of 100 rat units per day was maintained for the last eight and one-half days (table 2).[3] For details of standardization of ovarian and placental extracts reference is made to Allen and Doisy and others ('24).

OBSERVATIONS

Effects of injections in the living animals

Toward the end of the first week of injections a very light reddening of the skin of the vulva appeared in both animals.

[2] The third and fourth extracts were furnished by Parke, Davis Company, of Detroit. Their standardization in rat units was confirmed by the writer, who wishes to acknowledge the generous cooperation of Dr. A. W. Lescohier in supplying these extracts.

[3] No attempt was made to analyze the urine for excretion of hormone (Loewe and Lange, '26; Smith, '27, and others) for this would have required the restriction of the animals to small cages. It was thought better to allow them the range of a large outdoor cage. Consequently, although more than 1000 rat units were injected into each animal during the experiment, we have no assurance that all was utilized. It is possible that considerable amounts were excreted.

TABLE 2
Injections into immature monkeys 2E and SS

DATE	TIME	2E		SS		REMARKS
		cc. injected	R.U. daily	cc. injected	R.U. daily	
3/ 2/27	10.00 A.M.	1 LF 17		1 P no. 28		Injections begun
	4.45 P.M.	1	8	1	14	
3	9.00 A.M.	1		1		
	5.00 P.M.	1	8	1	14	
4	9.00 A.M.	1		1		
	5.00 P.M.	8	8	1	14	
5	8.30 A.M.	1		1		
	5.00 P.M.	1	8	1	14	
6	9.00 A.M.	1		1		
	5.00 P.M.	1	8	1	14	
7	9.00 A.M.	1		1		
	5.00 P.M.	1	8	1	14	
8	9.00 A.M.	1		1		Slight reddening of
	5.00 P.M.	1	8	1	14	'sexual skin'
9	9.00 A.M.	1		1		
	5.00 P.M.	1	8	1	14	
10	9.00 A.M.	1		1		
	5.00 P.M.	1	8	1	14	
11	9.00 A.M.	1		1		80 R.U. into 2E, 140 R.U.
	4.30 P.M.	1	8	1	14	into SS in first ten days
12	9.00 A.M.	0.5 P.D.		0.5 P.D.		Increased reddening and
	5.00 P.M.	0.5 (25 u)	25	0.5 (25 u)	25	swelling
13	9.00 A.M.	0.5		0.5		
	5.00 P.M.	0.5	25	0.5	25	
14	9.00 A.M.	0.5		0.5		75 R.U. in each in the
	5.00 P.M.	0.5	25	0.5	25	next three days
15	9.00 A.M.	0.5 P.D.		0.5 P.D.		
	5.00 P.M.	0.5 (100 u)	100	0.5 (100 u)		
16	9.00 A.M.	0.5		0.5		'Sexual skin' very red
	5.00 P.M.	0.5	100	0.5	100	and swollen
17	9.00 A.M.	0.5		0.5		
	4.30 P.M.	0.5	100	0.5	100	
18	9.00 A.M.	0.5		0.5		
	5.00 P.M.	0.5	100	0.5	100	
19	9.00 A.M.	0.5		0.5		'Sexual skin' phenomena
	5.00 P.M.	0.5	100	0.5	100	at maximum
20	9.00 A.M.	0.5		0.5		
	5.00 P.M.	0.5	100		100	
21	9.00 A.M.	0.5		0.5		
	4.30 P.M.	0.5	100	0.5	100	
22	9.00 A.M.	0.5		0.5		850 R.U. in each during
	5.00 P.M.	0.5	100	0.5	100	the last eight days
23	9.00 A.M.	0.5	50	0.5	50	Killed for histological study
Total,			1005		1065	

By the end of the second week of the experiment this had increased in intensity and had spread to the inner surfaces of the thighs and to the buttocks around the ischial calluses. The skin ventral to the vulva had a swollen or 'pouchy' appearance. On the twenty-second day of injections the reddened area was roughly circular, with an average diameter of 10 cm. The pouched or swollen skin ventral to the vulva was roughly triangular in shape, with a blunt apex toward the vaginal opening. In monkey 2E the base and altitude of this triangular area were 4.5 × 5.5 cm., while in monkey SS these were 2.25 × 4.5 cm. In control monkey SL and in three other young animals of nearly the same body weight there was no sexual differentiation of the skin of this region, the skin being tight and colorless.

Smear preparations of the vaginal contents of the injected animals during the latter half of the experiment showed increasing numbers of epithelial cells and decreasing numbers of leucocytes. The proportion of partially and completely cornified to nucleated epithelial cells increased during the latter half of the experiment. These results are similar to those obtained in ovariectomized adult monkeys.

Postmortem findings

Measurements of uteri. The three animals were killed for further histological study on March 23, 1927. In the experimental animals the uterus was hyperemic; in the control, anemic. Measurements were made of the diameters of the body of the uterus before removal of the organ and of the cervix after the genital organs had been dissected out. These measurements are listed in table 3.

A first control measurement of the body of the uterus (8.5 × 5.5 mm.) was obtained from animal LE at postmortem examination and a second control (11 × 7.5) from SS at ovariectomy. Compared with these, the average of the two postmortem measurements of injected animals SS and 2E (14 × 10.5 and 14 × 10 mm.) indicates an increase of slightly more than 4 × 3 mm. in diameter. There was an increase of

3×2.5 mm. in the uterus of monkey SS at the end of injections. In making this comparison, however, it should be noted that an eleven-day period of 'castrate atrophy' intervened between ovariectomy and the beginning of injections. The increase in cross-section area would of course be more than three times the increase in average diameter. Table 3 should be compared with tables V and XIX (Allen, '27) in which are listed measurements of uteri of mature animals.

Measurements of the cervices (table 3) show the average diameter of the injected animals to exceed the control by 6.5 mm.

TABLE 3

Measurements of diameter of uteri and cervices of control and experimental animals

MONKEYS	UTERUS	CERVIX	REMARKS
	mm.	*mm.*	
LE, control	8.5×5.5	11.0×9.5	Normal, no injections
2E, injected	14.0×10.5	17.0×15.0	After injections
SS, control	11.0×7.5	—	Before injections at ovariectomy
SS, injected	14.0×10.0	18.0×17.0	After injection at postmortem

Weights of endocrine glands. After removal of the reproductive organs, other endocrine glands were removed, trimmed of excess connective and adipose tissue, weighed, and fixed for histological study. These weights are listed in table 4.

TABLE 4

Weights of endocrine glands in grams

GLANDS	LE (CONTROL)	SS (OVARIECTOMIZED, INJECTED)	2E (NORMAL, INJECTED)
Two ovaries	0.224		0.092
Left suprarenal	0.393	0.330	0.339
Right suprarenal	0.326	0.284	0.263
Thyroids	0.252	0.260	0.402
Pituitary	0.059	0.045	0.046
Thymus	4.31	2.71	2.41

There is no significant difference in weights of suprarenal, pituitary, or thyroid glands of control and injected animals. The thymus glands and ovaries do, however, show significant weight differences. The thymus glands of injected animals, both the spayed and normal, are much smaller. This is in accord with the observations of Laqueur, Hart, and DeJongh ('26) upon decrease in thymus weights in rats which had been injected with 'menformon.' Arvin and Allen ('28) have confirmed this in dogs. The involution of the thymus has apparently been hastened in these two monkeys by injections of ovarian hormone.

The difference in weights of the ovaries is further supported by their sizes after fixation. An average for control ovaries was $7.5 \times 5.1 \times 3.8$ mm., for those of injected animal 2E, $5 \times 4.9 \times 2.2$ mm. These comparative weights and sizes seem to indicate a harmful effect of injected hormone upon the ovaries. This phase of the experiment will be discussed later after a histological description of the ovaries.

Histological examination of effects of injections

The mammary glands were removed, fixed in formalin, stained with dilute haematoxylin, and mounted as whole preparations, the nipples first being clipped off for study in sections. Sections were made of the reddened 'sexual skin' and the swollen areas ventral to the vulva. Cross-sections were made through the vaginal wall, the body of the uterus, the uterine tube at several levels, and the nipple. Sagittal sections were made through the cervix, and the ovaries were sectioned serially.

The sexual skin. The histology of the reddened and swollen 'sexual skin' of the mature monkey has been studied by Collings ('26). He concluded that the reddening was due to engorgement of the superficial blood vessels supplying this locality and that the swelling or 'pouching' was due to modified structure of the subepithelial tissues.

The skin and subcutaneous tissue down to the rectus abdominis muscle through the region of the 'pouching' was

4 mm. thick in control animal LE, as compared with 9 to 10 mm. in spayed injected animal SS and 12 to 13 mm. in normal injected animal 2E. These measurements were made after dehydration of the tissues, which produced considerable shrinkage. The other measurements of these swollen areas taken from the living animal have already been recorded.

The epithelium of these regions of the injected animals was composed of about twice as many layers of cells as that of the control (LE, control, 4 to 5; SS, spayed injected, 7 to 9, and 2E, normal injected, 8 to 10 layers). The sweat and sebaceous glands of the 'sexual skin' seemed to be more highly developed in the injected animals. The greatest change was apparent in the increased amounts of loose-meshed connective tissue containing extensive deposits of fat.

The vaginal wall. Control monkey LE had a vaginal epithelium which varied from four to eight layers in thickness (fig. 1). In some regions it was quite heavily infiltrated with leucocytes. Occasional mitoses were found in the basal layer, the plane of division being parallel to the basal membrane. There was no distinct stratum corneum. Leucocytes occurred more frequently in the outer layer. There were a few small epithelial bulbs growing down into the connective tissue (left center of fig. 1).

Injected ovariectomized animal SS had a vaginal epithelium which varied from twenty-six to sixty layers in thickness (fig. 2). There was a lower zone which stained darkly and a superficial zone composed of cells with pyknotic nuclei which had lost most of its affinity for basic stain. The cells of the superficial layer were much flattened and partly cornified. The basal border was indented between many epithelial bulbs and contained many cells undergoing mitotic division. Figure 20 shows the tip of one of these bulbs which contained an anaphase, two telophases, and several prophases of mitosis in a very limited area.

Normal injected monkey 2E also had a thick vaginal epithelium which was differentiated into two zones, although the distinction was not marked in some regions. The epithelium

averaged about thirty cells in thickness (fig. 3). Mitoses occurred frequently in the basal layers. Figures 1, 2, and 3 were all photographed at the same magnification ($\times$ 85) and are therefore directly comparable.

The cervix. Control monkey LE. The low stratified vaginal epithelium was continued around one side of the lip of the external os, where it made an abrupt transition to the single-layered epithelium of the cervical canal (fig. 4). This epithelium was quite heavily infiltrated with leucocytes and quite folded and indented by gland pits. In some regions the epithelium was low columnar, in others cuboidal. The glands were not well developed.

Spayed injected monkey SS. The thick stratified, partly cornified vaginal epithelium did not extend into the opening of the external os of the cervix (fig. 6). The single-layered epithelium of the cervical canal was very high columnar. It appeared pseudostratified in many regions. It was very heavily infiltrated with leucocytes. In many regions this epithelium had the 'brush border' characteristic of the epididymis. The glands were very well developed, and formed deep single pits or were branched at the ends. Heavy streams of polymorphonuclear leucocytes were present in the lumina of the glands and in the cervical canal. Some of the deeper glands were distended with secretion and comparatively free from leucocytes.

Normal injected animal 2E. The cervical glands were also well developed in this animal (fig. 5) but not so highly developed as in animal SS.

Figures 4, 5, and 6 are camera-lucida drawings made at the same magnification to show the relative development of the cervix, the thickness of the cervical epithelium, and the development of the cervical glands in control and experimental animals. Note the tortuous cervical canals.

The body of the uterus. Control animal LE. This uterus was small and anemic (see measurements listed in table 3). The lumen was slit-shaped and the glands were short and poorly developed. The epithelium of the deeper parts of

the glands was intact and contained occasional mitoses. The surface epithelium, however, was low cuboidal and distinctly degenerate in appearance in many regions (fig. 10). The tips of some of the folds of the endometrium were distinctly degenerated. It should be recalled that this uterus is from a normal immature monkey. This animal had consequently never menstruated and, judging from the body weight, the first menstrual period would probably not have occurred for six to twelve months. Consequently, these degenerative changes are probably not connected with menstrual processes.

Spayed injected animal SS. The uterus was much larger (table 3), the endometrium thicker, and the muscular walls very much better developed. The surface epithelium was clearly defined and showed no signs of degenerative changes. The glands were extremely long and showed considerable coiling and branching in the tips next to the internal muscle layer (figs. 8, 11, and 13). Mitoses were very frequent in both the surface epithelium and the glands. Figure 13 shows an area which contained five mitotic figures in a single section of a gland tip.

Normal injected animal 2E. The uterus of this monkey was even larger than that of spayed injected animal SS. The endometrium was thicker and the surface epithelium much greater in extent, having been thrown into rather high folds which made an irregularly shaped lumen (fig. 9). There was a narrow zone of lighter connective tissue underlying the epithelium which is very clearly shown in figure 9. The dark line marking it off was due to the subepithelial net of blood vessels. No very large blood vessels were to be found superficial to this line. It was present but less clearly defined in the uterus of animal SS (fig. 8). The glands of this endometrium had also begun to coil at the tips, as shown in figure 12. The incidence of mitoses was extremely high, as shown in figure 14 where at least nine can be counted in a single section of the tip of one gland.

The photographs of the uterus were all taken at the same level and at the same magnification ($\times$ 8), and are therefore directly comparable as to size.

The uterine tubes. Control tubes are represented by two specimens from normal animal LE and one tube from animal SS removed at the time of ovariectomy before injections were begun. Both showed a low columnar epithelium with a general absence of cilia (figs. 15 and 16).

Spayed injected animal SS. The second tube removed from this animal at the end of the series of injections showed a higher columnar epithelium in which mitoses were much more frequent. In some regions cilia appeared, while adjacent cells remained non-ciliated. (Compare fig. 17 with 16, the other tube from this same animal before injections.)

Normal injected animal 2E. The tubal epithelium of this animal was quite evenly ciliated, as shown in figures 18 and 19. Figure 18 is taken from a region in which a few droplets of secretion appear among the cilia, indicating the beginning of secretory activity by some of these epithelial cells.

The ovaries. Control ovaries are represented by two pairs from animals LE and SS. They showed a large number of follicles (approximately twenty) at a moderate stage of growth and distention with liquor folliculi. As would be expected, no corpora lutea were present. Sections through the longitudinal axes are shown in figures 21 and 22. The whole cortex of these normal ovaries was heavily laden with small follicles (primary ova). They were so abundant that any attempt at an estimation of their number would be extremely difficult (fig. 24).

The two ovaries of injected animal 2E were much smaller than the controls (see table 4 for weights). A further impression of the relative size may be had by comparison of figure 23 with figures 21 and 22, all of which are median sections through the longest dimension of the ovaries. The number of moderately well-developed follicles was about one-half (ten) that of the control, and the follicles were less well developed than in the control ovaries. The cortex of the ovaries from the normal animal contained relatively few primary ova. Comparison of figures 24 and 25 brings out this difference clearly.

4 mm. thick in control animal LE, as compared with 9 to 10 mm. in spayed injected animal SS and 12 to 13 mm. in normal injected animal 2E. These measurements were made after dehydration of the tissues, which produced considerable shrinkage. The other measurements of these swollen areas taken from the living animal have already been recorded.

The epithelium of these regions of the injected animals was composed of about twice as many layers of cells as that of the control (LE, control, 4 to 5; SS, spayed injected, 7 to 9, and 2E, normal injected, 8 to 10 layers). The sweat and sebaceous glands of the 'sexual skin' seemed to be more highly developed in the injected animals. The greatest change was apparent in the increased amounts of loose-meshed connective tissue containing extensive deposits of fat.

The vaginal wall. Control monkey LE had a vaginal epithelium which varied from four to eight layers in thickness (fig. 1). In some regions it was quite heavily infiltrated with leucocytes. Occasional mitoses were found in the basal layer, the plane of division being parallel to the basal membrane. There was no distinct stratum corneum. Leucocytes occurred more frequently in the outer layer. There were a few small epithelial bulbs growing down into the connective tissue (left center of fig. 1).

Injected ovariectomized animal SS had a vaginal epithelium which varied from twenty-six to sixty layers in thickness (fig. 2). There was a lower zone which stained darkly and a superficial zone composed of cells with pyknotic nuclei which had lost most of its affinity for basic stain. The cells of the superficial layer were much flattened and partly cornified. The basal border was indented between many epithelial bulbs and contained many cells undergoing mitotic division. Figure 20 shows the tip of one of these bulbs which contained an anaphase, two telophases, and several prophases of mitosis in a very limited area.

Normal injected monkey 2E also had a thick vaginal epithelium which was differentiated into two zones, although the distinction was not marked in some regions. The epithelium

plasm remained. The cell membrane was very indistinct. The arrangement of the surrounding follicle cells was completely disorganized.

This follicle illustrates three progressive stages of elimination of ova from polyovular follicles. I have not seen a description of this process in a follicle so far advanced in development as the one described.

Another type of follicular atresia which was present in all the ovaries of this series, but much more marked in the ovaries of injected animal 2E, is illustrated in figures 27 to 29. These structures appear to be atretic follicles which have collapsed or been flattened after reaching a considerable stage of development with formation of liquor folliculi. The right-hand structure in figure 28 contains a degenerating ovum in its center, while that on the left still has a small lake of liquor folliculi. It is hard to determine whether the scar represents a transformed follicular epithelium or a modified theca. The cells are often spindle-shaped and similar to typical connective-tissue cells. Figures 27 and 29 show advanced stages of this same sort of atresia. These scars are interpreted as flattened remains of follicles which presumably were formerly moderately well developed.[4] Their high incidence in the ovaries of injected animal 2E would account for the great decrease in number of moderately large follicles so obvious in comparison of figure 23 with figures 21 and 22.

The mammary glands. There is considerable variation in the growth of mammary glands of immature monkeys. Consequently, it seems desirable that, where injection effects are under consideration, one gland be used for a specific control and the other for an experimental gland. Figure 35 is a camera-lucida silhouette of a flat mount of the left gland of animal SS removed at the time of ovariectomy. It is composed of seven major ducts which branch freely and altogether have approximately 1000 terminal alveoli. After

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[4] The literature on atretic follicles is so great that I have been unable at this time to search extensively for descriptions of this type of atresia. I have not, however, seen this latter type described.

removal of the control gland, there was a period of twelve days before injections were begun during which some castrate atrophy might have been expected. The second (right) mammary gland was removed thirty-three days after the first at the end of a twenty-one-day series of injections, during which 1065 rat units of ovarian hormone were injected (fig. 36). A small section of this gland was clipped out for histological study. The remainder had approximately 2300 terminal alveoli, more than twice the number in the control.

The epithelium of the nipple of the experimental gland was somewhat thickened. For comparison with injection effects upon the mammary glands of mature animals, reference is made to figures 44 to 51 and text figures A to D (Allen, '27).

SUMMARY OF HORMONE EFFECTS

1. More than 1000 rat units of ovarian hormone were injected into each of two monkeys; a third of the same body weight served as a control. One was normal with ovaries intact, the second was ovariectomized before injections were begun.

2. Reddening and swelling of the 'sexual skin' was induced in both normal and spayed immature monkeys.

3. The ovaries and the thymus glands of injected animals weighed much less than those of the controls. No significant weight differences were noted in other endocrine glands.

4. The epithelium of the vaginal wall was greatly thickened. Rapid hyperplasia was indicated in the basal layers.

5. Cervical and uterine measurements indicated considerable growth in the uterus. Sections show greatly thickened endometrium, hyperplasia of surface epithelium and glands, and proportionate development of the muscular walls.

6. The tubal epithelium of the control monkeys was for the most part non-ciliated. In the spayed injected animal there are frequent patches of rudimentary cilia. In the normal injected animal ciliation is quite uniform and secretion droplets are present in some regions.

7. The ovaries of the injected animal were much smaller than those of the controls. They contained relatively few follicles—both primary and moderately well-developed follicles with liquor-folliculi formation. The incidence of polyovular follicles was high. A polyovular follicle quite far advanced in development containing four ova in successive stages of atresia is described. A second type of atresia interpreted as flattening or collapse of formerly moderately large follicles, resulting in an unusual sort of scar formation, is described. The incidence of these peculiar follicle scars is higher in the ovaries of the normal injected animal and may account for the smaller number of moderately large follicles in these ovaries.

8. There was growth in the right mammary gland of the spayed injected animal, resulting in more than twice the number of terminal alveoli as had developed in the left (control) gland.

CONCLUSIONS

1. Ovarian hormone is responsible for 'sexual skin' characteristics in monkeys.

2. Its principal action is to induce growth of the genital tract and mammary glands.

3. There seems to be a specific inhibitory effect upon the thymus gland. No significant weight differences were noted in other endocrine glands.

4. In this concentration and over this period of time this hormone apparently inhibits growth of follicles (both moderately well-developed and primary) and accentuates the process of selective elimination. (More animals should be used before this conclusion is considered definite.) At least, there is no evidence from these experiments that this ovarian hormone should be used in human cases of infantilism.

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PLATE 1

Figures 1, 2, and 3 Cross-sections of vaginal walls at a magnification × 85.

1 Normal control monkey LE. Very thin epithelium four to eight cells in thickness with few epithelial bulbs and only occasional mitoses.

2 Spayed injected monkey SS. Very thick epithelium differentiated into two zones having many epithelial bulbs and many mitoses in the basal layer (see fig. 20).

3 Normal injected monkey 2E. Thick vaginal epithelium, similar to that of spayed monkey SS, but not so well developed.

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PLATE 2

Figures 4, 5, and 6   Camera-lucida drawings of sagittal sections of the cervix in control and experimental animals, drawn at a magnification $\times 8$ and reduced to $\times 4$.

4   Normal control monkey LE.

5   Normal injected animal 2E.

6   Spayed injected animal SS.

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Figures 7, 8, and 9 are sections through the body of the uterus below the junction of the uterine tubes. Magnification $\times$ 8.

7 Control animal LE. Uterus small and anemic, lumen slit-shaped, glands poorly developed.

8 and 9 Spayed injected animal SS and normal injected animal 2E. Much thickened endometrium with clearly defined surface epithelium and well-developed glands which have grown until they have begun to coil and branch at their tips. Note the contrast in development of the muscle layers of the uterine walls with those in figure 7.

Figures 10, 11, and 12   Sections of the endometrium, showing higher magnification ($\times$ 85) of figures 7, 8, and 9, respectively.

10   Control monkey LE.   Glands small but composed of normal columnar epithelium containing some mitotic figures.   Surface epithelium low cuboidal and with degenerate appearance in many regions.

11 and 12   Spayed injected monkey SS and normal injected monkey 2E.   Tips of greatly elongated glands shown in figures 8 and 9, respectively.
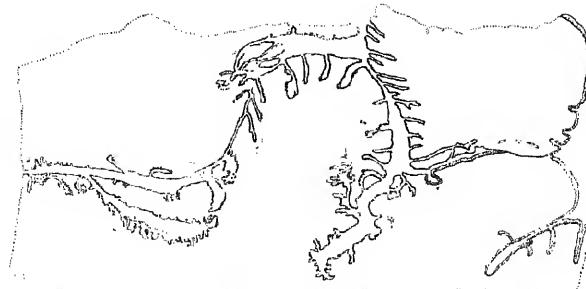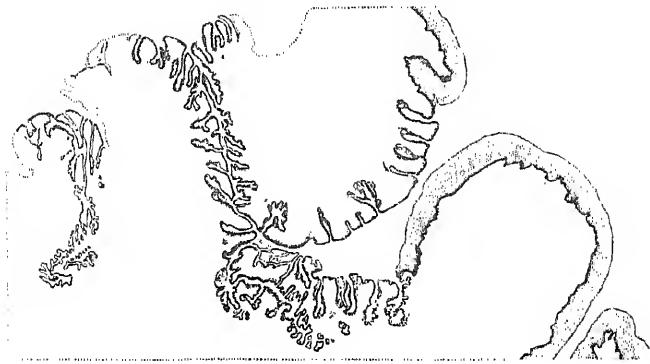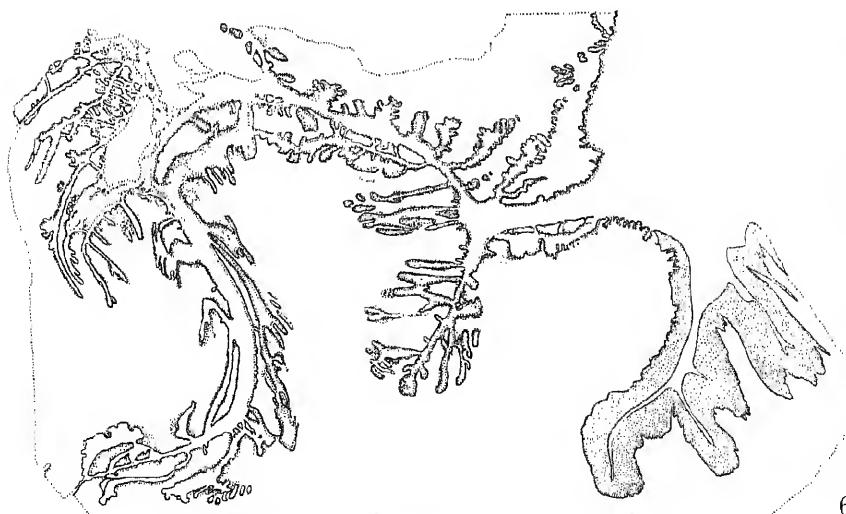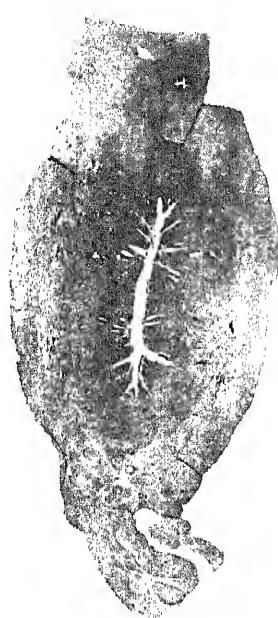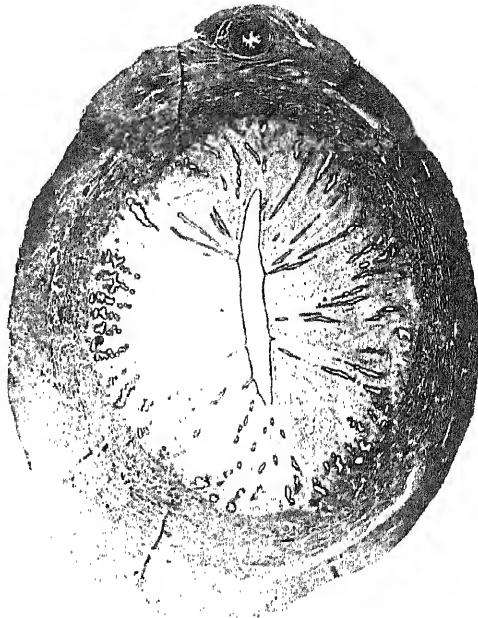
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Figures 7, 8, and 9 are sections through the body of the uterus below the junction of the uterine tubes. Magnification $\times$ 8.

7 Control animal LE. Uterus small and anemic, lumen slit-shaped, glands poorly developed.

8 and 9 Spayed injected animal SS and normal injected animal 2E. Much thickened endometrium with clearly defined surface epithelium and well-developed glands which have grown until they have begun to coil and branch at their tips. Note the contrast in development of the muscle layers of the uterine walls with those in figure 7.

21, 22, and 23 Sections of ovaries through the greatest diameters; magnification × 12.

Figures 21 and 22 are normal control ovaries from monkeys SS (before injections) and LE; figure 23 is from injected normal monkey 2E. Note the difference in number and degree of development of moderately large follicles.

24 and 25 Sections of normal ovary of monkey SS (from fig. 21) and injected monkey 2E (from fig. 23) at a higher magnification (× 85) to show the great contrast in the number of small follicles (primary ova).
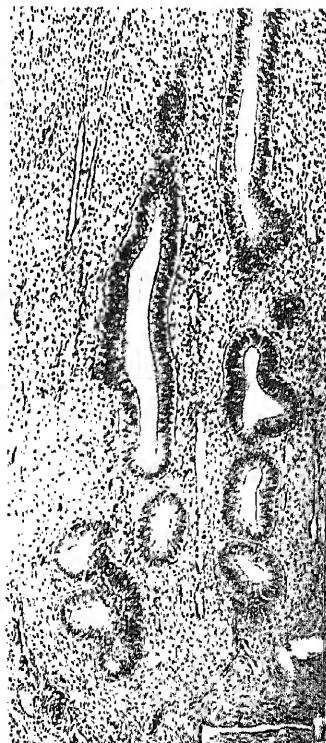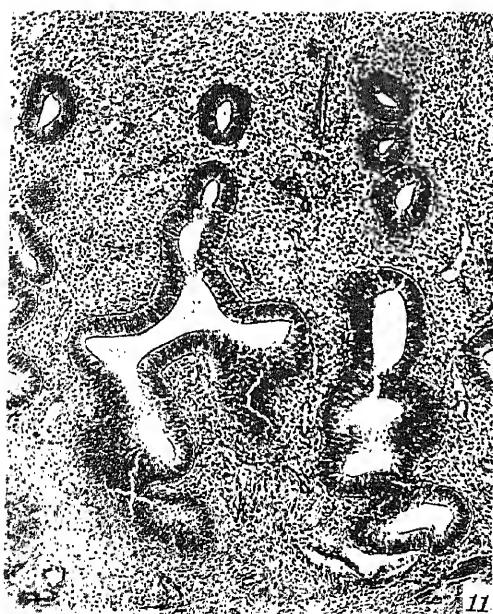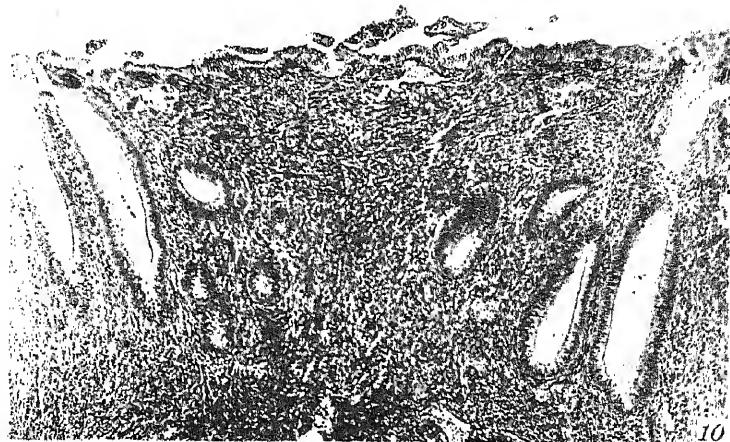
35   Whole mount of left mammary gland after removal of the nipple; from animal SS at the time of removal of the ovaries; mounted flat.

36   Right mammary gland from the same animal thirty-three days later, after injection of 1065 rat units of hormone. There are approximately twice as many terminal alveoli in this gland.
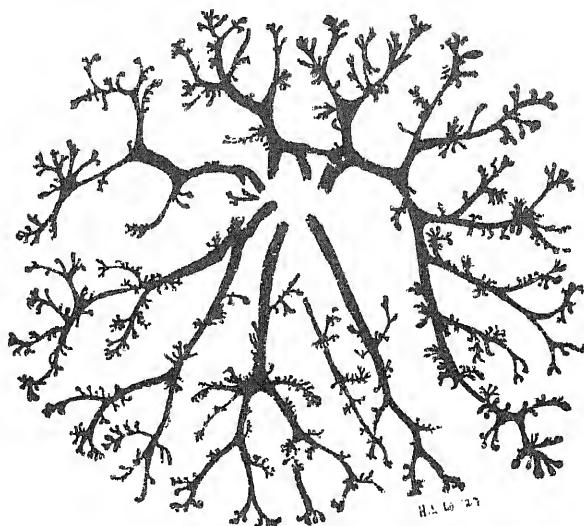
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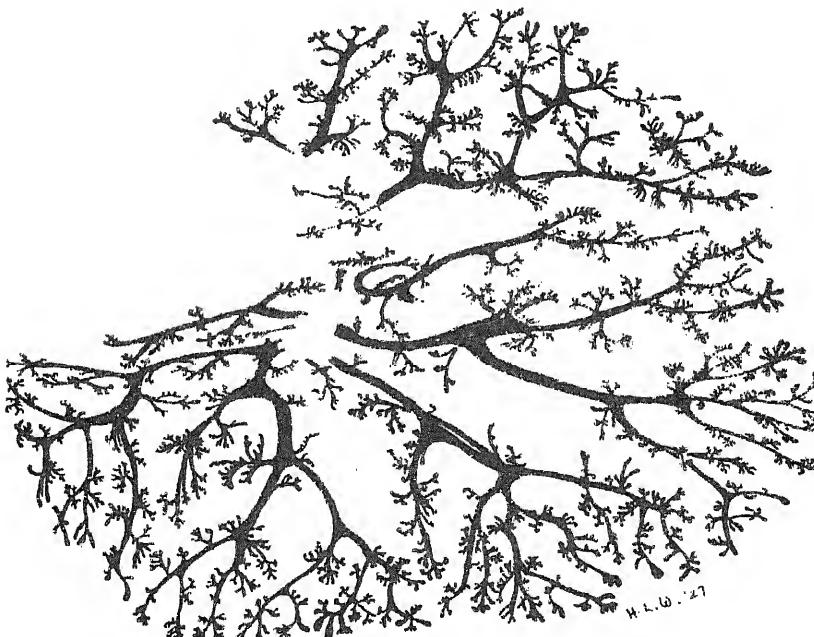
BINARY FISSION AND ENDOMIXIS IN THE TRICHODINA FROM TADPOLES (PROTOZOA, CILIATA)[1,2]

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ONE TEXT FIGURE AND FOUR PLATES (FORTY-NINE FIGURES)

AUTHOR'S ABSTRACT

This peritrichous ciliate lives as an ectocommensal on the skin and gills of anuran tadpoles. Its relation to described species of Trichodina is doubtful. This study was made almost exclusively on fixed and stained material. Binary fission is similar to that in other ciliates. The horseshoe-shaped macronucleus condenses, then divides amitotically. The single small micronucleus forms a spindle containing between four and six chromosomes.

Endomixis is of high incidence in the free-living Trichodinae. Encystment was not observed. At the onset of endomixis, the macronucleus disintegrates into fragments which persist throughout the process. The micronucleus undergoes three rapidly succeeding mitotic divisions to form eight nuclei. There is no evidence of chromosome reduction during these divisions. Seven of the nuclei differentiate into macronuclear anlagen; the eighth becomes the functional micronucleus. Successive cell divisions—before each of which the micronucleus divides—distribute macronuclei to daughter cells. Variations from the regular process of endomixis may arise, 1) by precocious division of endomictic parents; 2) by extra divisions of the micronucleus; 3) by less than the usual number (three) of divisions of the micronucleus; 4) by hypertrophy and early differentiation of the micronucleus into macronuclei; 5) by unusual segregation of nuclei to daughters, and, 6) from miscellaneous causes.

The significance of these variations is discussed in connection with the possible origin of bimicronucleate and amicronucleate races.

CONTENTS

[1] A thesis in zoölogy presented to the faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] An abstract of this paper has appeared in The Anatomical Record, vol. 34, 1926.

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INTRODUCTION

Endomixis in ciliates has opened an attractive field for investigation since the classic work of Woodruff and Erdmann ('14), describing and interpreting the cytological details of the process in Paramecium aurelia. It was suspected at that time that this phenomenon was of general occurrence in the Infusoria. However, the literature dealing with the morphological changes during endomixis is exceedingly meager. Erdmann and Woodruff ('16) extended their previous observations with an account of endomixis in P. caudatum, showing that it .corresponded closely to the same phenomenon in P. aurelia, and that the essential difference between conjugation and endomixis was the fact of synkaryon formation in the former process and its absence in the latter. Probably some of Hertwig's ('89) conjugation stages of P. aurelia were really isolated cases of endomixis. Recently, Erdmann ('27) described endomixis in P. bursaria and stated that the process was similar to that in P. caudatum, but few details were given. Woodruff and Spencer ('23), in a brief paper describing P. polycaryum, sp.nov., mention the occurrence of endomixis in this species. Calkins ('15) showed one figure of endomixis in Didinium nasutum and later ('19) announced the discovery of endomixis in Uroleptus mobilis while the animals were encysted. Moore ('24 a) described and figured endomixis in Spathidium spathula, also during encystment. She states in another paper ('24 b)—without giving any further description—that "there are several indications that Blepharisma, like Paramecium, undergoes periodic nuclear reorganization during vegetative life." Klee ('26) has interpreted as endomixis a reorganization process which occurs in both the active and encysted stages of Euplotes longipes. The new macronucleus was described as being reconstituted from part of the old macronucleus. Since micronuclear anlagen which should build a new macronucleus were not found, this account seems incomplete. Fermor ('13) described in the cysts of Stylonychia pustulata a process of nuclear reorganization which involves autogamy and is therefore not to

be interpreted as endomixis. His findings have not been confirmed. Recently, Manwell ('28) has described a nuclear reorganization, during encystment, in Pleurotricha bifaria. He says: "A remarkable feature of encystment is the extrusion of the old macronuclei, and perhaps one of the micronuclei, through the cyst wall. After this has happened the normal nuclear complex is rebuilt from the single micronucleus remaining. Whether the presence of the latter is regularly due to the extrusion of the other micronucleus from the cell, or to the fusion of the two original micronuclei is not altogether certain."

This extrusion of nuclear material reminds one of the 'budding' of Councilmania cysts. Ivanić ('28) has also reported a nuclear-reorganization process in the cyst of Chilodon uncinatus. Although some of Ivanić's interpretations may be doubted, it is clear that extensive nuclear changes of an endomictic nature occur in this ciliate.

The present studies have been undertaken in order to contribute to the information relating to the details of endomixis, as exhibited in the Trichodina from tadpoles. The following is the first account of endomixis in the peritrichs and, as far as the writer is aware, the first description of endomixis in an associated ciliate.

MATERIALS AND METHODS

The subject of these studies is the Trichodina which lives as an ectocommensal on the skin and gills of anuran tadpoles. All the species of tadpoles examined (Rana catesbiana,

R. clamitans, R. palustris, R. sylvatica, R. pipiens, and Bufo
sp.sp.) harbored at least some Trichodinae. There was ap-
parently no host specificity in relation to the tadpoles. No
indications of a strictly parasitic relationship between the
Trichodina and the tadpoles were observed, although it was
noticed that the smaller tadpoles often supported a greater
population of Trichodina than the larger ones. This small
size, however, cannot be construed as positive evidence of a
pathogenic effect on the part of the Trichodina.

Tadpoles were collected from two general sources : 1) ponds
and streams in the vicinity of Philadelphia and, 2) ponds in
the environs of Lancaster, Pennsylvania. The Trichodinae from
these two sources were found to agree in their specific identity,
although they were racially different. The Philadelphia race
of Trichodina was larger, had a greater number of denticles
in its corona and a smaller micronucleus than the Lancaster
race. On account of the larger micronucleus and the exceed-
ingly heavy incidence of the Lancaster Trichodinae, they have
been used almost exclusively as the material for this paper,
although the same type of reproductive phenomena occurs in
both groups.

The observations have been made on fixed and stained ma-
terial. No serious attempts have been made to culture Tricho-
dina artificially. In almost all cases the tadpoles were used
very soon after they had been brought into the laboratory, in
order that the Trichodina on them might be fixed under as
nearly natural conditions as possible. The internal gills were
exposed, extracted, placed in a small drop of water on a
cover-slip, and teased apart with needles. The cover-slips
were then inverted onto the surface of the fixative. These
operations were performed as rapidly as possible, since the
body fluids of the host are exceedingly toxic to the Tricho-
dinae. The gills were regularly used, in preference to the
skin, on account of the convenience in handling and because
the albuminous material expressed from them helped to
fasten the Trichodinae closely to the cover-slip. Generally,
two slides were procured from each tadpole—one from each

set of gills. About 750 preparations of this kind were made. Warm and cold Schaudinn's fluid, with and without acetic acid, a modification of Bouin's fluid, weak and strong Flemming's, Zenker's, and Hollande's fluids were used as fixatives. The preparations were stained with Heidenhain's iron haematoxylin, Mayer's haemalum, Delafield's haematoxylin, or Mallory's triple stain and sometimes counterstained with orange G or eosin. The best results were obtained by fixing in warm Schaudinn's fluid plus acetic acid, staining with Heidenhain's iron haematoxylin, and counterstaining with orange G.

OBSERVATIONS

A. General morphology and taxonomy

For a thorough review of the literature relating to the structure and systematic position of Trichodina, the reader is referred to Fulton's ('23) admirable paper. Trichodina has been classified by Calkins ('26) as follows: Subphylum Infusoria, Class Ciliata, Order Peritrichida, Family Vorticellidae, Subfamily Urceolarinae.

In shape the Trichodina from tadpoles resembles a turban with a narrow thick brim (fig. 2). The Lancaster race averages about 35 μ in diameter. The height of the turban is subject to considerable variation, depending on the substratum and the degree of activity of the animals. The base, by means of which the Trichodina glides specter-like over its host, is modified into an attaching structure, the sucking disk. Leaving aside the theoretical derivation of the dexiotropic adoral zone of cilia in the majority of the peritrichs and the attempted explanation of the apparent longitudinal division in forms like the Vorticellidae (Bütschli, '86, and Calkins, '26), descriptions in the following account will refer to the functional surfaces of the animal. The surface which functions as the attaching disk will be designated as the proximal side, the crown of the 'turban' as the distal surface, the side on which the mouth is located, the oral surface, and the side opposite the latter as the aboral surface. Extending from

the edge of the attaching disk is a narrow, almost transparent, membrane-like structure, the velum (fig. 2, *vel.*). Near the base of the velum lies the circle of basal granules for the proximal wreath of cilia (fig. 2, *pr.z.*). These cilia are very long and numerous.

The distal surface is bounded by a double row of cilia (fig. 2, *ad.z.*) which commences near the mouth, traverses the vestibule, and thence winds around the body in a counterclockwise direction—when the observer is looking at the proximal side— and ends slightly distal to the vestibular opening. The vestibule (figs. 3 and 4, *v*) is a spacious funnel-shaped cavity which inclines toward the side and communicates with the gullet through the cytostome (figs. 3 and 4, *o*); the gullet (figs. 3 and 4, *g*), an elongated passage, narrowing as it progresses inward and reaching to about the center of the body, takes a sharp turn at the place where it adjoins the vestibule. The food, apparently, consists exclusively of bacteria. The food vacuoles (figs. 1 and 3, *f.v.*) are spindle-shaped and limited to the central endoplasm. There is a single large contractile vacuole (figs. 2 and 4, *c.v.*).

Probably the most conspicuous feature of the fixed and stained Trichodinae is a ring of denticles, known as the corona (figs. 1 and 2, *C*). It lies just inside the concave attaching disk. The chemical nature of the substance composing the corona is not known. The rigidity and concomitant flexibility required of the attaching disk by which the animals adhere to and creep along irregular surfaces are supplied by the corona. It consists of a number of separate denticles which fit so closely into one another as to give, sometimes, the appearance of a solid structure. Each denticle is equipped with two projections, a 'hook' which extends peripherally or outward, and a 'ray,' pointing centrally or inward. The number of denticles in the coronas of different individuals varies considerably. The smallest number observed in an adult animal was fifteen, while the largest number was thirty-four. It is likely that this number fluctuates about a single mode and that there are no racial differences involved in this variability.

For example, the numbers of denticles in the coronas of 372 individuals from one host were counted and the mode for this population was found to be twenty-one. The results are shown in the following table:

Number of denticles,	17	18	19	20	21	22	23	24	25
Number of individuals,	1	2	28	85	122	80	41	8	5

It was suspected that the number of denticles in the corona might increase, by interpolation, with the age of the individual. That this possibility is not a valid one and that the number of denticles remains constant during the life of an individual is suggested by further analysis of the above data. Of the 372 animals ·examined, thirty-five were recently divided individuals. In this group of thirty-five the largest class consisted of those individuals in whose coronas there were twenty-two denticles—a higher number than the mode for the entire population.

Immediately above (distal to) the corona and separate from it, is a system of radiating lines which extend to the periphery (figs. 1, 2, 6, 25, and 44, r.s.). They, doubtless, lend additional support to the sucking disk. There are about five or six of these radial striations to each denticle. Very often, peripheral thickenings appear on alternate radiations (fig. 44).

The nuclear apparatus consists of a large horseshoe-shaped macronucleus (figs. 1 and 2, M), the open end of which incloses the vestibule, and a single small micronucleus (figs. 1 and 2, m). The macronucleus exhibits typically a heterogeneous structure, perhaps best described as pocketed. With the Schaudinn fixation and the Heidenhain staining, numerous black granules of varying sizes and staining capacities appear suspended, inside little clear pockets, in a more faintly staining finely granular matrix. Haemalum-stained material gives a different effect. The matrix stains very much as it does by the Heidenhain technique, but the granules inside the pockets do not take the stain. It is quite evident, therefore, that the material composing these granules is different

from the chromatin of the matrix. At times, during vegetative existence, macronuclear material is found lying free in the cytoplasm. This represents material which has been extruded from the surface of the macronucleus. Sometimes these extrusions are comparatively large fragments of the body of the macronucleus, while at other times they are merely the intrapocketal granules.

The micronucleus, in the resting condition, appears vesicular in structure and is generally ellipsoidal. It is regularly located near the end of the macronucleus, between it and the cell membrane (on the left as seen in fig. 1, m), but very rarely may be found lying on the opposite side of the body. All the chromatic material of the micronucleus is concentrated into a central endosome which is separated by a clear space from the delicate nuclear membrane. The entire vesicle measures somewhat less than 3 μ in length. Individuals possessing two micronuclei (fig. 3, m) and also individuals without any visible micronucleus have been found occasionally. The significance of these forms will be discussed later.

Fulton ('23), in his review of the taxonomy of the genus Trichodina, recognizes only three species: T. pediculus Müller (Ehrb.), T. steinii Clap. et Lach., and T. urinicola Fulton. It is probable that the Trichodina from tadpoles is none of these species and so far, a search of the literature has failed to confirm its specific position. Accordingly, the species to which the Trichodina from tadpoles belongs must be left undetermined for the present.

B. Binary fission

Although a number of investigators have carefully described the behavior of the corona of Trichodina during division, the only description of the nuclear changes attendant upon division has been given in Russian by Peshkowsky ('23) for T. steinii and T. mitra (Urceolaria mitra). Therefore, an account of the division phenomena of the Trichodina from tadpoles would seem to be desirable. When the animal is preparing to divide, the horseshoe-shaped macronucleus con-

denses to a more compact body. It loses its regular smooth contour and presents a rather wavy outline (figs. 4 and 5). Instead of being pocketed, its composition changes to a condition of fine uniform granularity. The staining capacity of the macronucleus, at this time, increases. As condensation progresses, the chromatin tends to become disposed in parallel strands so that the macronucleus has a somewhat striated appearance (fig. 5). The form of the macronucleus becomes more and more irregular, giving the impression of a thick twisted cloth. It may finally become so compact as to be almost spherical (fig. 7). At the time of its maximum condensation, fragments from the macronucleus are often found scattered through the cytoplasm (figs. 5, 6, 7). Their significance is problematical. The macronucleus then divides, becoming dumbbell-shaped (fig. 8), and then pulling apart into two halves (fig. 9).

Probably the whole process of binary fission is initiated by the activity of the micronucleus. During the prophases it swells, becomes spindle-shaped, and reaches a maximum length of about 6 μ. The endosomal mass gives rise to the spindle, chromosomes, centrioles, and a centrodesmose. By the time the macronucleus has become finely granular and is just beginning to condense, a well-defined metaphase spindle has usually formed (figs. 4 and 6), although the synchrony of the division of the macronucleus and micronucleus is subject to considerable variability, as an inspection of the figures will show. Chromosome counts are difficult to make on account of the small size of the nucleus, but it is certain that the number is small—four, five, or six. During the anaphase the two groups of well-clumped daughter chromosomes separate and migrate toward the poles. As migration continues, a separation spindle forms (figs. 7 and 8), as it commonly does in ciliates, and within this the centrodesmose is frequently well defined. When separation is complete, the daughter micronuclei are about 12 μ apart. The separation spindle may persist until constriction of the cell body sets in. It is of some interest to note that material originally inside

the micronucleus and later composing part of the separation spindle becomes liberated into the cytoplasm at each cell division.

At the time when the macronucleus starts to constrict, the new definitive corona of the prospective daughters has made its appearance in the parent cell. There is a narrow, apparently unsegmented ring midway between the old corona and the circumference of the disk (figs. 6, 8, and 9, C_2). This ring is believed to contain the fundaments of the denticles for the coronas of the daughters.

The history of the other cytoplasmic organelles during division was not followed with much assurance. Peshkowsky ('23) stated that "during the process of division in Trichodina steinii and T. mitra the adoral zone, the throat and the contractile vacuole become absorbed." In the Trichodina from tadpoles, however, it is likely that the old cytopharynx undergoes some dedifferentiation and becomes the functional gullet of one of the daughters, while the aboral part of the old peristome differentiates into the new cytopharynx of the other daughter.

Of some interest is the formation in the parent of a system of vacuoles (figs. 7 and 9, *vac.*) lying near the aboral surface in the plane of constriction, one of which maintains a definite opening to the outside through a short tube. It is suggested that these vacuoles serve to expedite constriction of the cell body by lessening the viscosity of the cytoplasm at that place. Whether any of these vacuoles develop into the functional contractile vacuole of one of the daughters was not determined. The parental contractile vacuole probably persists in one of the daughters.

The constriction of the cell body starts on the distal surface and continues proximally toward the attaching disk (figs. 9 and 10). Division of this type is the rule in the vorticellids, if the stalked end of forms like Vorticella is regarded as homologous with the disk of Trichodina. This apparent longitudinal division has been brought into line with the transverse fission in other ciliates by assuming a shift in polarity.

As the attaching disk is pinched into two, the old corona is divided into two parts in such a way that each piece contains approximately the same number of denticles. In the daughters the broken ends round up so as to form almost complete circles again (figs. 10, 11, and 12, C_1). The peripheral precoronal ring, which has likewise been divided between the daughters, segments and rapidly develops 'hooks' and 'rays.' The number of denticles in the new corona is determined at a very early stage in the life of the animal—sometimes in the parent before division—and persists without change during its existence. About twice as many denticles are developed in the new outer corona as are present in the old inner half corona. Stein ('64) observed two-ringed specimens of Trichodina pediculus, apparently, and gave them the name Trichodina diplodiscus. The inner corona becomes less and less conspicuous and is finally resorbed, while the outer corona develops and takes its place. Just how long the old corona persists is not known. Probably the time is comparatively short. Cases have been seen in which the new corona had been fully formed and the old one had practically disappeared before the separation of daughters had been completed. The possession of two coronas, an outer one with the regular number of denticles and an inner one with one-half that number in it, is a very convenient criterion for identifying recently formed daughters.

The daughter macronuclei at first are ellipsoidal and faintly staining. They generally contain a comparatively small number of large granules, connected by a chromatinic meshwork, and lying in a clear matrix. As the ellipsoid lengthens into the curved adult nucleus, its chromaticity increases and the pocketed structure is regained (figs. 10, 11, and 12).

C. Normal process of endomixis

Under this term Woodruff and Erdmann ('14) described "a complete periodic nuclear reorganization without cell fusion in a pedigreed race of Paramecium." "The internal nuclear phenomena comprise the formation of a complete new

nuclear apparatus of micronuclear origin.'' Endomixis, as
thus defined, was of very frequent occurrence in the Tricho-
dina from tadpoles. Scarcely a slide failed to furnish at least
some stages in the process. That epidemics of endomixis
occur in the population of Trichodina is suggested by the
observation that on a single slide more individuals are likely
to be in a certain stage of endomixis than in other stages.
Moreover, endomixis was more frequently encountered in the
late spring, especially in June, than at any other time of the
year. Whether the generally more favorable environmental
conditions in the spring, for protozoan life, or the develop-
mental habits of the tadpoles was the responsible factor,
could not be determined. Endomixis takes place while the
animals are in the free-living condition. No encystment has
ever been observed.

The onset of endomixis is characterized by the breaking up
of the macronucleus (fig. 13, M'). In most cases the macro-
nucleus breaks up completely by forming numerous spherical
bodies of varying sizes. Each fragment represents an origi-
nal pocket or pockets of the macronucleus, containing a deeply
staining granule lying in a clear space surrounded by a
finely granular chromatic shell. The outer part of these frag-
ments disappears first, leaving behind the central granule
surrounded by a faint halo. Finally, this loses its staining
capacity and disappears also. These fragments, at the start
of endomixis, are closely packed and preserve the horseshoe
shape of the intact nucleus (figs. 13 and 40, M'). Later, they
are carried by cytoplasmic currents throughout the entire
endoplasm. In some cases the macronucleus breaks up into
a relatively small number (two, three, four, or five) of large
irregular pieces, each of which soon separates into the char-
acteristic 'haloed' particles. The disappearance of the old
macronuclear fragments is not complete until after the new
nuclear apparatus has been fully reconstituted.

The micronucleus migrates from its normal position—as
it is seen in figure 1 (m) on the left side of the body near the
vestibule—toward the aboral side of the animal (fig. 13, m).

Here it completes its first division. Figure 14 (m') shows a metaphase of this first division. This stage is difficult to iden-tify because the deeply staining macronuclear fragments tend to obscure the micronucleus. During the migration of the micronucleus, the endosome lengthens, becomes curved, and develops the spindle inside the nuclear membrane. The endo-mictic spindles are apparently similar to those occurring dur-ing binary fission, except that the former may be a little larger. The reaction of the cell, as a whole, to the division of the micronucleus is interesting. By the time a well-defined metaphase spindle has developed, a new corona is formed outside the old corona—as far as the observations go—even though the cell does not divide (figs. 14 and 15, C_2 and C_1). The old corona persists at least until the end of the second division and is then resorbed. This behavior suggests an abortive attempt at cell division which does not go to .com-pletion. The two nuclei resulting from the first division of the micronucleus immediately divide again, giving rise to four nuclei. Figure 15 shows two early anaphases of this division. These four nuclei are larger than the resting vege-tative nucleus and they have a deeply staining angular endo-some, prophetic of the third and last division. Figure 16 (m') shows the four spindles in mitotic division. One is in early anaphase, while the other three are still in metaphase. The nucleus on the left side, nearer the vestibule, regularly divides before the rest. Figure 17 shows a later stage in the third division. The nuclei on the left have completed their division, while the two on the right are in early telophase stages. In the few cases in which it has been possible to count chromosomes, during endomictic nuclear division as in figures 16 and 27 (m'), the number was the same as in ordi-nary vegetative division.

The final eight products of the micronuclear divisions are originally all apparently similar. Seven of them, however, rapidly differentiate into macronuclear anlagen, while one remains the functional micronucleus (figs. 18 and 19). These eight body stages are the ones most frequently found in the

study of endomixis. Characteristically, the nuclei are arranged in two groups. Four nuclei are located on the right side of the body and the other four on the left side. The regular position of the functional micronucleus is shown in figure 18 (m_2), where it is seen on the left side between the first and second macronuclear anlagen from the vestibule. Sometimes, however, it may be situated between the second and third anlagen and even, though rarely, on the opposite side of the body. The future functional micronucleus does not undergo any modification, but remains small and undifferentiated.

Differentiation of the macronuclear anlagen is effected by increase in size and loss of chromaticity. They first enlarge, but retain their dense, deeply staining capacity. Very often they become lemon-shaped (fig. 18, M_2) and there is always a clear space inside the nuclear membrane. In correlation with their further increase in size, the macronuclear anlagen lose their chromaticity. This is accomplished in a number of ways. Chromatic granules, often of considerable size, may appear on the surface. Sometimes, they are limited to one pole, or both poles, or they may be scattered irregularly over the surface. These chromatic granules separate from the body of the nucleus and are extruded into the cytoplasm. In other cases vacuoles are formed, inside the nuclei, in which a kind of autodigestion of chromatic material seems to be effected. Occasionally, the macronuclear anlagen during this reorganization are flat and leaf-like, pointed at the ends, and curved. Finally, the texture of the anlagen assumes a condition of fine granularity (fig. 19).

The extent to which the macronuclear anlagen have differentiated is not rigidly fixed by the time the first cell division is inaugurated. Generally, at this time they are still spherical or slightly ellipsoidal, about 6 µ in diameter, and still in the process of chromatic reorganization. The micronucleus moves toward the periphery and divides (figs. 19 and 21, m_2), as it does before each succeeding cell division. By a constriction of the cell membrane two daughters are formed, one of

which receives four macronuclear anlagen and a micronucleus, while the other receives three macronuclear anlagen and a micronucleus (figs. 20, 21, and 22, M_2 and m_2). In these daughters the differentiation of the macronuclear anlagen continues. Before the next cell division they have generally acquired their final pocketed structure and have begun to lengthen out into the characteristic adult form. Figures 20 and 21 show the anlagen in the granular condition. The micronucleus of the individual represented in figure 21 (m_2) is in metaphase, preparing for the next division. The macronuclear anlagen of the animal illustrated in figure 22 (M_2) are much further differentiated than those of figure 21. The micronucleus of the former is in the prophase stage.

The individual with four macronuclear anlagen and a micronucleus gives rise to two daughters, each with two macronuclear anlagen and a micronucleus (fig. 23). Each one of these animals then divides again, so that the final postendomictic cells each have a single macronucleus and a micronucleus (fig. 24, M and m). The daughter which has three macronuclear anlagen as the result of the first cell division divides, forming one daughter with a single macronucleus and a micronucleus and another daughter possessing two macronuclear anlagen and a micronucleus. The bimacronucleate individual divides once more before reorganization is complete, segregating a single macronucleus and a micronucleus to each daughter. By the time the bimacronucleate condition is reached the old macronuclear fragments have largely disappeared. The anlagen stain very faintly, exhibiting a sparse chromatic groundwork supported in a clear non-staining matrix, suggestive of the rapid intake of fluid.

The sequence of events in this process is shown diagrammatically in text figure A (p. 536).

D. Variations in the normal process of endomixis

Striking variations from the usual scheme, outlined above, have been encountered. Representative cases are illustrated on plates 3 and 4. For the sake of convenience, these varia-
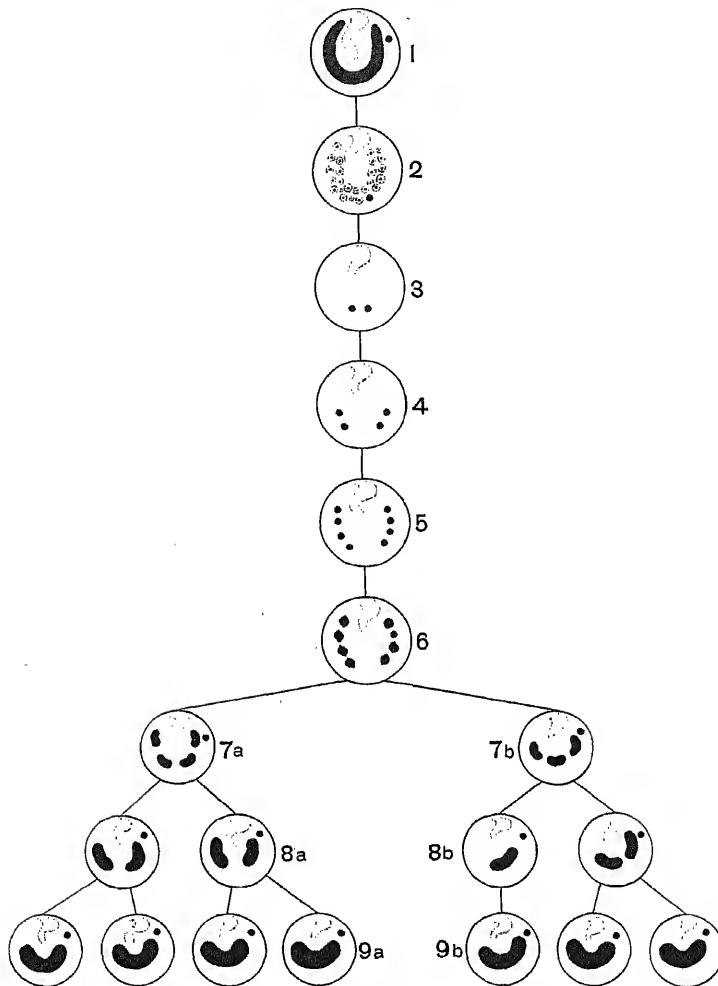
Text fig. A Diagram of the normal process of endomixis. *1*, Normal vegetative animal; *2*, macronucleus fragmented and disintegrating, and not shown in subsequent diagrams. Micronucleus has migrated to the other end of the body; *3*, micronucleus divided first time; *4*, micronucleus divided second time; *5*, micronucleus divided third time; *6*, seven of the nuclei differentiating into macronuclear anlagen, while the eighth remains the functional micronucleus which divides before each cell division; *7a* and *7b*, daughters resulting from the first cell division and having four and three macronuclear anlagen, respectively; *8a* and *8b*, daughters resulting from the second cell division. The *8b* monomacronucleate individual is completely reorganized; *9a* and *9b*, daughters resulting from the third cell division. Growth of the macronucleus will reconstruct them into normal vegetative individuals.

tions may be classified in the following manner: 1) variations resulting from precocious division of endomictic parents; 2) variations resulting from 'extra' (more than three) divisions of the micronucleus; 3) variations arising from less than the usual number (three) of divisions of the micronucleus; 4) variations resulting from the early differentiation of the micronucleus; 5) variations arising from an unusual segregation of nuclei to daughters; and, 6) miscellaneous variations, involving disintegration, lagging behind, or unusual division of certain nuclei. These different categories are not necessarily mutually exclusive.

1. *Variations resulting from precocious division of endomictic parents.* Figure 25 shows a recent daughter (distinguished by having two coronas, the outer one with twenty-two denticles and the inner one with ten) which has probably arisen from an endomictic parent whose micronucleus had divided for the first time. This division resembles that which takes place during ordinary binary fission, except that the macronucleus has fragmented. This individual might possibly be interpreted as an animal initiating endomixis immediately after a vegetative cell division. The fact that there are hardly enough macronuclear fragments to account for a complete macronucleus, however, militates against this interpretation.

It is difficult to identify beyond question individuals which have resulted from precocious division of endomictic parents, because the normal number of nuclei in animals during various stages in the regular process includes such a wide range —one, two, three, four, or eight. However, a number of cases observed in which the anlagen were in an exceptionally immature condition might fall in this category.

2. *Variations resulting from 'extra' (more than three) divisions of the micronucleus.* Figures 26 to 38 illustrate this type of phenomenon. Figures 26 and 27 give a clue to the origin of this situation. In the individual represented in figure 26 there are four large nuclei and four small ones. If the group of larger nuclei should have divided again, as they

probably would have done, a twelve-nucleate individual would have resulted. Figure 27 shows an individual, a number of whose nuclei are in various stages of the fourth micronuclear division. If the two large nuclei in the upper right center should continue to divide, a fifteen-nucleate animal would be formed. Individuals with such a nuclear complex have been found. Figure 28 illustrates one of these cases in which the functional micronucleus has divided preparatory to the first cell division, giving sixteen nuclei. In the two preceding cases the micronucleus became segregated after the third nuclear division. In figure 29 (m_2) is shown a case in which the micronucleus was not determined until after the fourth division. It is in prophase, preparing for the first cell division. The segregation of supernumerary macronuclear anlagen to daughter cells cannot be predicted with any degree of certainty. That some of these supernumeraries, at least, are viable and capable of division is shown in figure 30. This is a young daughter possessing an inner corona of eight partially disintegrated denticles, a new outer corona beginning to form, seven macronuclear anlagen, and a micronucleus. In every respect it is a normal-appearing animal.

A variety of other supernumerary-nucleated conditions is possible. Figure 31 shows an animal with twenty-seven nuclei. Three of these are significantly larger than the rest and possibly they alone would have developed into the functional macronuclear anlagen. One of the remaining twenty-four, different in shape and staining capacity, may be the functional micronucleus. Figure 32 illustrates an individual having twenty-three nuclei, of which four—two on each side—are micronuclei. Three of the remaining nineteen nuclei are different from the rest. Whether they are going to degenerate or are merely lagging behind cannot be decided. It seems probable that two micronuclei would have been segregated to each of the subsequent daughters and that after final reorganization a bimicronucleate race would have arisen. Such may have been the origin of the bimicronucleate animal shown in figure 3. Figure 33 represents an individual with nine nuclei, four of

which appear as typical macronuclear anlagen, four are degenerating nuclei, and the remaining one is the micronucleus. Figure 34 illustrates an individual with ten nuclei. This condition has probably arisen by an extra division, in the eight-body stage, of the two nuclei on the right side next to the vestibule. All of these nuclei appear to be normal and capable of further development. Figure 35 shows an individual with eleven nuclei, some of which have arisen by extra divisions. Four of them are functional macronuclear anlagen, one is a large micronucleus preparing for division, and the remaining six small nuclei are in early stages of degeneration. Figure 36 illustrates a twelve-body stage. Such an individual probably resulted, as was suggested in connection with the description of figure 26, from an extra division of four of the nuclei in the eight-body stage. Here it is impossible to predict the fate of these twelve seemingly similar nuclei. In other cases, as for example in figure 37, there is more difference among the supernumerary nuclei and it is possible to predict their fate with more assurance. It is likely that this condition has arisen by an extra division, in the eight-body stage, of three of the young macronuclear anlagen. The resulting six small nuclei are consequently either lagging behind the four large anlagen, which did not divide in the eight-body stage, or are destined to degenerate. In the meantime, the functional micronucleus has divided into two small dense daughter micronuclei, in anticipation of the first cell division. In figure 38 is shown an animal with thirteen nuclei. One of them is the dividing micronucleus and three of the macronuclear anlagen are considerably larger than the rest. Probably four of the nuclei in the eight-body stage underwent an extra division and then one of these eight nuclei divided once more.

To what extent the vegetative bimicronucleate stock may have been responsible for the origin of supernumerary-nucleated endomictic individuals and whether the bimicronucleate stock persists even through endomixis, are questions which have not been solved. It is felt, however, that the very few bimicronucleate vegetative animals which have been found

could account for only a small proportion of the 'extra'-
nucleated endomictic individuals, even if the supernumerary
conditions were of the same type as would be expected to
have arisen from a bimicronucleate race.

*3. Variations arising from less than the usual number
(three) of divisions of the micronucleus.* In contrast to the
condition in which there may be 'extra' nuclear divisions, a
number of cases have been found in which the usual eight
nuclei formed from three divisions of the original micronu-
cleus have not materialized. Figure 39 shows one of a number
of instances of this sort. Here there are seven nuclei, six of
which are dense and homogeneous, while the seventh has
started to differentiate into a macronuclear anlage. Prob-
ably this individual arose by the failure of one (not the one
destined to give rise to the functional micronucleus) of the
nuclei to divide in the four-body stage, while the other three
continued to divide again. In other cases the nucleus which
is going to be the functional micronucleus fails to divide in
the four-body stage, while the others continue to divide.
Another instance, not illustrated, points to the possibility of
two of the nuclei in the four-body stage failing to divide while
the other two continued to do so, thus giving rise to a six-
nucleate condition.

*4. Variations resulting from the early differentiation of the
micronucleus.* Rarely, the micronucleus hypertrophies and
then divides. Figure 40 (*m''*) shows one of these hypertro-
phied nuclei beginning to divide. Chromatin masses, scat-
tered irregularly, but somewhat concentrated at each end
of the nucleus, suggest an attempt at chromosome formation.
Figure 41 (*m''*) represents a later stage in the division of
such a hypertrophied nucleus. Figure 42 (*m''*) shows the two
large nuclei resulting from this division. Figure 43 gives
evidence that these nuclei are functional, for a time at least,
and are capable of division. One of them has divided entirely
and the other is completing its division. It is very likely that
these four nuclei are capable of dividing another time, but
whether they would continue functional after final reorgani-

zation cannot be determined. In the event of persisting viability of this sort of individual it is interesting to speculate as to whether there would be a dedifferentiation of a nucleus to form a micronucleus, or whether an amicronucleate race would arise. Figure 44 shows an individual with two coronas of approximately at least—some of the denticles of the inner corona have been resorbed—the same number of denticles and four differentiating nuclei. This double corona, as in the normal process of endomixis, suggests an abortive division on the part of the cell. Two possibilities as to the origin of this condition present themselves: first, that these nuclei arose from differentiating nuclei like those represented in figures 40 to 43, or secondly, that they arose from four originally undifferentiated nuclei. In view of their small size the latter alternative seems more likely.

5. *Variations arising from an unusual segregation of nuclei to daughters.* A number of individuals possessing five macronuclear anlagen have been encountered. One of them is drawn as figure 45. The most logical interpretation of this condition, since no degenerating anlagen could be found, is to assume that in the eight-body stage instead of four macronuclear anlagen going to one daughter and three to the other daughter, the segregation was in the nature of a five-and-two allotment. The micronucleus in the case selected is in the prophase of division. Another possible interpretation of this animal is that there was a suppression of the division of a nucleus, or nuclei, in the two-body or the four-body stage.

6. *Miscellaneous variations.* Figure 46 illustrates an endomictic individual with a micronucleus, three functional macronuclear anlagen, and two degenerating nuclei. This six-nucleate condition could have arisen either as the result of unequal segregation of anlagen in the eight-body stage, or from the eight-body stage itself with the complete disappearance of two more nuclei, or as the result of the failure of certain nuclei to divide in the early stages of endomixis.

Figure 47 shows a reorganizing individual in which one macronuclear anlage is lagging considerably behind the other

two. However, this retarded anlage is perfectly normal to all appearances, and there is no reason to suppose that it would not continue to function.

Figure 48 represents an individual which has six macronuclear anlagen and two micronuclei, instead of the usual seven-and-one arrangement. This condition could have arisen by the division of one of the nuclei in the four-body stage, not into a functional micronucleus and a macronuclear anlage, but into two micronuclei. The three other nuclei could have divided normally into six anlagen.

A number of other variations have been met in the course of these studies, but the examples given above are typical and indicate the wide range of variability which is possible in the process of endomixis in the Trichodina from tadpoles.

DISCUSSION

Binary fission in the Trichodina from tadpoles corresponds closely to similar phenomena in other ciliates. However, the behavior of the macronucleus and the formation of a separation spindle by the micronucleus are worthy of note. The changes in the disposition of chromatic material from the pocketed condition, to the granular condition, to the form of parallel strands, and then in the reverse direction suggest that there is more than a haphazard method in the division of the macronucleus. The significance of the formation of macronuclear fragments extruded during condensation can only be conjectured.

Among all the animals studied, only three unquestioned cases of conjugation have been seen. In these the conjugants were of the same size—at least they were not more unequal than ordinary neighbors were. It may be of interest to compare briefly the behavior of some of the Vorticellidae during conjugation. In Vorticella there is a highly specialized anisogamy. In Urceolaria patellae (= Trichodina patellae Cuénot) Caullery and Mesnil ('15) have reported that the only recognizable difference between the two kinds of gametes is one of size, there being no morphological modification.

The microgametes are represented as being about one-half as large in diameter as the macrogametes. The nuclear details, as reported, are so closely similar to those of endomixis in the Trichodina from tadpoles that it is suspected that endomixis has been confused with conjugation. Peshkowsky ('23) has stated that "the conjugation of Trichodina steinii is apparently equal to that of Vorticellidae in all its essential features." She also shows figures of what is probably endomixis in a form she calls Trichodina mitra (= Urceolaria mitra), but which are interpreted as conjugation. In the free-swimming vorticellids and in the Trichodina from tadpoles there is complete isogamy. It is, therefore, not possible at present to correlate the method of conjugation of the Vorticellidae with their mode of life.

There is little danger of confusing conjugation with endomixis in the Trichodina from tadpoles—even if there were a greater incidence of the former—because, in the cases observed, the macronucleus disintegrates in a distinguishingly different way in conjugation from the way it does in endomixis. The macronuclear fragments, instead of being spherical as in endomixis, are rectangular, linear, or spindle-shaped. Woodruff and Erdmann ('14) and Woodruff and Spencer ('22) reported similar differences in the macronuclear disintegration of Paramecium aurelia. They found that macronuclear disintegration was effected typically in endomixis by the elimination of spherical chromatin bodies, instead of by the transformation of most of the macronucleus into long tangled chromatin ribbons, such as occurs during conjugation.

Conjugation always results in physical reorganization of the protoplasm. The old macronucleus is broken up and the fragments are absorbed by the cytoplasm, while a new macronuclear and micronuclear apparatus is differentiated from products of the first, second, or third division of the synkaryon after fertilization. A similar periodic reorganization (thirty days for P. aurelia and sixty days for P. caudatum) of the nucleus is accomplished by endomixis. The essential morphological difference between conjugation and endomixis

is the presence of synkaryon formation in the former and its absence in the latter process. Opposing ideas as the physiological value of these phenomena in the Infusoria have been reviewed by Calkins ('26) and Woodruff ('25). Both endomixis and conjugation—when they occur—seem to have the same physiological effect in renewing the vitality and prolonging the life of the race. Calkins has interpreted a life-cycle in the ciliates, comparable to that in the Metazoa, characterized by periods of youth, maturity, and old age. He believes that the continued vitality of protozoan lines, under natural conditions, depends on the intervention of conjugation, endomixis, encystment, or some other reorganizing process, to restore the senile, worn-out protoplasm to its youthful labile condition. Woodruff, however, does not grant that the decrease in vitality is due to any intrinsic aging tendency in the protoplasm, but believes that the 'life cycle' has been induced by improper artificial-culture methods, and that neither endomixis nor conjugation is indispensable to the continued existence of ciliates. He ('25) says: "Weighing all the available facts the conclusion seems justified that when degeneration is in progress endomixis has a rejuvenating effect, and we thus reach the general result that *both* conjugation and endomixis have a similar effect under similar conditions—both meet the emergency of physiological degeneration *induced by environmental conditions* which are not ideal."

Since no attempt was made to study pedigreed lines of Trichodina, the effect of endomixis on its life-cycle and its dispensability or indispensability on the continued existence of the race were not learned. The very great incidence of endomixis would seem to point to its practical value in the economy of the life of the species. Moreover, one wonders whether the macronuclear material extruded during intermitotic periods and the nuclear fragments frequently separated from the condensed macronuclear mass during binary fission might not have some effect on the vitality of the race.

In attempting to homologize endomixis in the ciliates with similar processes in the Metazoa, the suggestion has been

made by Calkins ('26) and others that endomixis is, in reality, parthenogenesis. Since the continuity of nuclear material from generation to generation is maintained through mitotic divisions of the micronucleus, it is logical to consider the micronucleus as equivalent to the female gamete of the Metazoa which develops without fertilization. Previous accounts of endomixis have not positively eliminated the possibility of autogamy from the process, but in the present studies no evidence of micronuclear fusion has ever been found. The question then arises as to whether chromosome reduction is effected during endomixis. It has been stated that, during binary fission, the diploid number of chromosomes falls within the limits of four and six. If reduction of chromosomes were to occur in endomixis, it would necessarily have been accomplished by the time the metaphase of the third micronuclear division had been reached. In the few cases, for example, in figures 16 and 27, in which it was possible to study the chromosomes during endomictic divisions the diploid number was counted. It is legitimate, therefore, to interpret endomixis as parthenogenesis of the diploid type.

All of the eight nuclei resulting from the three divisions of the original micronucleus are apparently similar as they are first formed. They are of the same size, all have the vesicular type of structure, and it is impossible, for a short time, to distinguish the future functional micronucleus from the macronuclear anlagen. The later relative constancy in the position of the micronucleus on one side of the body between the first and second macronuclear anlagen from the vestibule (left side of figs. 18 and 30, m_2) and the apparent absence of a differential micronuclear division lead one to infer that the determination as to which nucleus is going to be the definitive micronucleus is controlled by its location in the cell. Differentiation of the macronuclear anlagen, as stated before, is accomplished by the loss of chromatic material and by a tremendous increase in size. The micronucleus, in contrast, is not appreciably modified. This differential behavior implies the fate of the nuclei. The original eight nuclei may be thought of

as containing both idiochromatin and trophochromatin. Seven of them, apparently, get rid of their idiochromatin by extrusion, etc., develop their trophochromatin, and become vegetative, transient macronuclei. The eighth retains its idiochromatin and becomes the functional micronucleus, with reproductive potentialities.

The history of the eight nuclei in the endomixis of Trichodina is very different from that described for Paramecium. Woodruff and Erdmann ('14) state that in P. aurelia all but one or two of the eight micronuclear products degenerate. The cell then divides and each daughter receives a micronucleus. From this single micronucleus the macronuclear anlagen and the functional micronuclei are said to be derived. Only one more cell division is required to reconstitute the animals completely. In P. caudatum Erdmann and Woodruff are inclined to feel that, of the four micronuclei in the stage where there are four macronuclear anlagen and four micronuclei, two of the latter undergo degeneration. In Trichodina in the normal process of endomixis, there is no degeneration of nuclei. By reason of the uneven number, seven, of macronuclear anlagen, their segregation is unequal and the daughters which receive them must undergo an unequal number of divisions before reconstruction is complete. This is shown in the asymmetrical diagram of the process (text fig. A).

That the onset of endomixis is a critical period in the life of the organisms concerned is indicated by Erdmann and Woodruff's difficulty in maintaining, by the method of single daily isolations, their race of P. caudatum during endomixis. This is true also for Euplotes longipes and P. bursaria. During such a period of delicate readjustment it would be expected that the well-ordered sequence of events might sometimes be upset. The large number of variations from the general rule of endomictic behavior in Trichodina, some of which are figured, shows that this nice balance is sometimes disturbed. The quadrimicronucleate individual illustrated in figure 32 could well represent the ancestor of a bimicronucleate race, one of which is shown in figure 3.

On the other hand, some of the endomictic variations discovered may be responsible for amicronucleate races. Figures 36 and 40 to 44 show endomictic individuals which lack a discrete micronucleus and which—should they be viable—might have initiated amicronucleate races. Frequently, Trichodinae have been examined in which a micronucleus could not be found. It is by no means certain that all these were amicronucleate individuals, because the micronucleus may have been obscured by the overlying corona or by food vacuoles, etc., but without doubt some of them were cases of true amicronucleation. Prandtl ('06), in his study of the cytology of conjugation in Didinium nasutum, observed examples of the transformation of all the micronuclei into macronuclei, though he was doubtful if the animals with a nuclear heritage of this character were viable. Patten ('21), however, has studied a pedigreed culture of an amicronucleate race of Didinium nasutum which arose from conjugating micronucleate individuals. A number of other amicronucleate races have been reported: Dawson ('19) described a race of Oxytricha hymenostoma without a micronucleus. He bred it successfully in pedigreed cultures for several years and found that, although it attempted conjugation, merely abortive results followed. Landis' ('20) 'amicronucleate' race of Paramecium caudatum proved to be P. multimicronucleata, with four small micronuclei. Woodruff's ('21) 'amicronucleate' race of P. caudatum might also have been P. multimicronucleata. Amicronucleate races of Oxytricha fallax and Urostyla grandis were also reported by Woodruff ('21). Thon ('05) made a careful cytological study of Didinium nasutum, but was unable to discover a micronucleus in his strain. Moody ('12) carried on a long culture of Spathidium spathula which revealed no micronuclei. It is difficult to believe that either of these latter investigators could have overlooked micronuclei had they been present. The evidence derived from the study of Trichodina lends weight to Woodruff's ('21) speculation that, "it is possible—perhaps probable when we recall the observations of Prandtl and of Patten—that

under certain exigencies during cell reorganization the typical course of events may be disturbed so that the differential micronuclear division is suppressed or not precise and all the micronuclei metamorphose into 'macronuclei.' Such 'macronuclei' may be interpreted as amphinuclei. There is, a priori, no reason why nuclei of this character should not be adequate for the typical life phenomena of the cell, except probably those involving endomixis or conjugation. Such apparently is the potentiality of the nuclear apparatus of the amicronucleate races thus far described.''

Erdmann's ('20) findings that endomixis in Paramecium aurelia endows the race with distinct heritable variations and that after endomixis lines with greater or less differences in size appear suggest the possibility of similar phenomena in Trichodina. Here there is abundant morphological evidence that during endomixis nuclei frequently digress from their well-ordered path of behavior, and after doing so, are segregated into postendomictic daughters. Such unusual chromatic endowments are probably a sufficient physical basis to account for phenotypic differences between different lines.

SUMMARY

1. The subject of these studies is the Trichodina living as an ectocommensal on the skin and gills of anuran tadpoles. The body is turban-shaped and the concave base is modified into an attaching disk, supported by a ring of denticles, the corona. The nuclear apparatus consists of a large horseshoe-shaped macronucleus and a single small micronucleus. The observations were made on fixed and stained material.

2. During binary fission of this Trichodina the micronucleus enlarges and becomes spindle-shaped. The endosome which fills most of the nucleus appears to give rise to the chromosomes, spindle fibers, and centrodesmose. A metaphase plate is formed in which four to six (the exact number is undecided) chromosomes lie. Each chromosome divides and the two groups of daughter chromosomes move to opposite poles of the nucleus. Constriction of the nuclear membrane follows

and the daughter micronuclei pull apart, being connected for some time by the separation spindle containing a centrodesmose. The horseshoe-shaped macronucleus loses its characteristically pocketed structure, becomes more finely granular, and later is traversed by longitudinal parallel chromatic strands. It condenses into an ovoid or spherical mass, then elongates and constricts in the middle. A new corona is formed external to the old corona. Constriction of the cell membrane divides the parent cell into two daughters, each of which has a macronucleus, a micronucleus, one-half the old corona, and the rudiments of a new corona. In the daughters the old coronas are resorbed, the new ones develop denticles, and the macronuclei regain their pocketed structure and their horseshoe shape.

3. A very few cases of conjugation have been found. These can be distinguished from endomixis by the different methods. of macronuclear disintegration in the two processes.

4. During the normal process of endomixis the macronucleus fragments into spherical particles—each containing a deeply staining central granule separated by a clear space from the limiting nuclear material. These particles persist through the entire process of endomixis. The micronucleus migrates from its usual position near the vestibule, toward the aboral side. Here it undergoes three successive mitotic divisions to form eight nuclei. Simultaneously with the first of these divisions, a complete new outer corona may sometimes be formed. This activity is considered to represent an abortive division on the part of the cell. Of the eight nuclei formed by the divisions of the original micronucleus, seven differentiate into macronuclear anlagen and one remains the functional micronucleus which divides at each succeeding cell division. As a result of the first cell division, four macronuclear anlagen and a micronucleus are segregated to one daughter, while three macronuclear anlagen and a micronucleus pass to the other daughter. In the former case two subsequent cell divisions are required for the distribution of macronuclear anlagen to daughters, so that each postendo-

mictic individual has only one macronucleus. In the latter case—in which the daughter has three macronuclear anlagen and a micronucleus—the next division segregates one macronucleus to one daughter and two macronuclear anlagen to the other daughter. The latter divides once more into two cells, each of which has a macronucleus and a micronucleus.

5. There is evidence that chromosome reduction does not take place during endomixis.

6. A number of variations from the normal process of endomixis can occur. These may be classified as follows: 1) variations resulting from precocious division of endomictic parents; 2) variations resulting from 'extra' (more than three) divisions of the micronucleus; 3) variations arising from less than the usual number (three) of divisions of the micronucleus; 4) variations resulting from the hypertrophy and early differentiation of the micronucleus; 5) variations arising from an unusual segregation of nuclei to daughters; and, 6) miscellaneous variations, involving disintegration, lagging behind, or unusual division of certain nuclei. These different categories are not necessarily mutually exclusive.

7. The suggestion is made that some of these variations are the bases of the origin of bimicronucleate and amicronucleate races.

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EXPLANATION OF PLATES

All figures in the plates are from preserved and stained material and have been outlined with a camera lucida. The animals have been fixed in Schaudinn's fluid, stained with Heidenhain's haematoxylin, and drawn at a magnification of 1800. They have been reduced one-half in reproduction. The figures have been oriented with the vestibule toward the bottom of the plate and, unless otherwise stated, with the attaching disk turned toward the observer. The cilia and coronas have been omitted for the sake of simplicity, except when they were thought to have special bearing on the subject in hand.

ABBREVIATIONS

m, micronucleus

M, macronucleus

C, corona

m', micronucleus dividing in endomixis

M', fragments of macronucleus in endomixis

m_2, new micronucleus

M_2, macronuclear anlagen

m'', hypertrophied micronucleus

C_2, new-generation corona

C_1, old-generation corona

$c.v.$, contractile vacuole

g, gullet

o, cytostome

v, vestibule

$vel.$, velum

$vac.$, vacuole

$ad.z.$, adoral zone of cilia

$pr.z.$, proximal zone of cilia

$r.s.$, radial striations

$f.v.$, food vacuole

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Figures 1 to 3, 'resting' individuals. Figures 4 to 12, stages in binary fission.

1 Typical vegetative individual. Cilia omitted.

2 Same in side view.

3 Bimicronucleate individual.

4 Macronucleus beginning to condense, wavy in outline, and finely granular. Micronucleus in metaphase. Centrodesmose visible. Centrioles probably shrunken away from the nuclear membrane. Attaching disk turned away from the observer.

5 Macronucleus traversed by longitudinal chromatic strands. Micronucleus in anaphase. Centrodesmose visible.

6 Further condensation of the macronucleus. Clear region surrounding the chromatin mass is probably a shrinkage space. Macronuclear fragments scattered through the cytoplasm. Micronucleus in metaphase. Precoronal ring has appeared.

7 Macronucleus in maximum condensation. Two nuclear fragments in cytoplasm. Micronucleus in early telophase. Centrodesmose visible. Vacuole toward aboral side.

8 Macronucleus beginning to pull apart. Well-formed separation spindle through which centrodesmose extends. Precoronal ring visible.

9 Micronucleus divided. Constriction of macronucleus almost complete. Vacuoles at aboral side.

10 Daughters almost separated. A large macronuclear fragment in right-hand individual. Macronuclei finely pocketed.

11 Recently formed daughter. New outer corona of twenty-one denticles acquiring hooks and rays. Old inner corona has ten denticles.

12 Slightly older daughter. Macronucleus assuming adult pocketed structure. Outer corona of twenty-one denticles well formed. Inner corona disintegrating.

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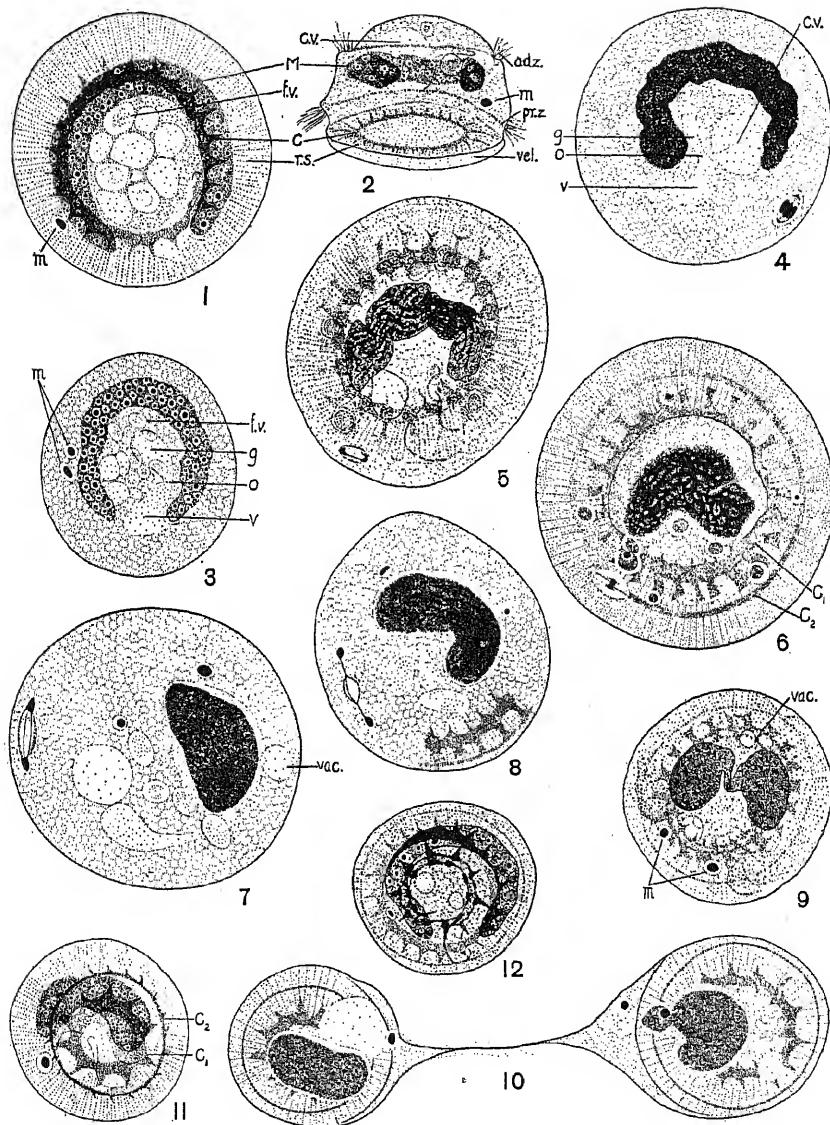
PLATE 2

Figures 13 to 24, stages in the normal process of endomixis.

13 Macronucleus fragmented into characteristic spherical bodies, each containing a deeply staining central granule. Micronucleus in aboral position, preparatory to first division.

14 First division of micronucleus; two coronas, each with twenty-one denticles. Attaching disk turned away from the observer.

15 Second nuclear division; two coronas, each with twenty-five denticles.

16 Third nuclear division; nucleus on the left in a later stage than the other three.

17 Late third division. The two nuclei on the left have already divided, while the two on the right are in late anaphase.

18 Early eight-body stage, showing seven darkly staining lemon-shaped macronuclear anlagen, of which the one on the lower left is furthest differentiated, and a micronucleus.

19 Late eight-body stage, showing seven differentiating macronuclear anlagen and a dividing micronucleus.

20 One of the daughters resulting from the first cell division; four well-differentiated macronuclear anlagen and a micronucleus.

21 Another daughter resulting from the first cell division; three slightly differentiated macronuclear anlagen and a micronucleus in metaphase.

22 Three pocketed macronuclear anlagen in a late stage of differentiation and a prophase micronucleus.

23 Individual resulting from the second cell division, having two macronuclear anlagen, a micronucleus, and two coronas.

24 Reorganizing animal, the result of the last cell division; a single macronucleus, a micronucleus, and two coronas.
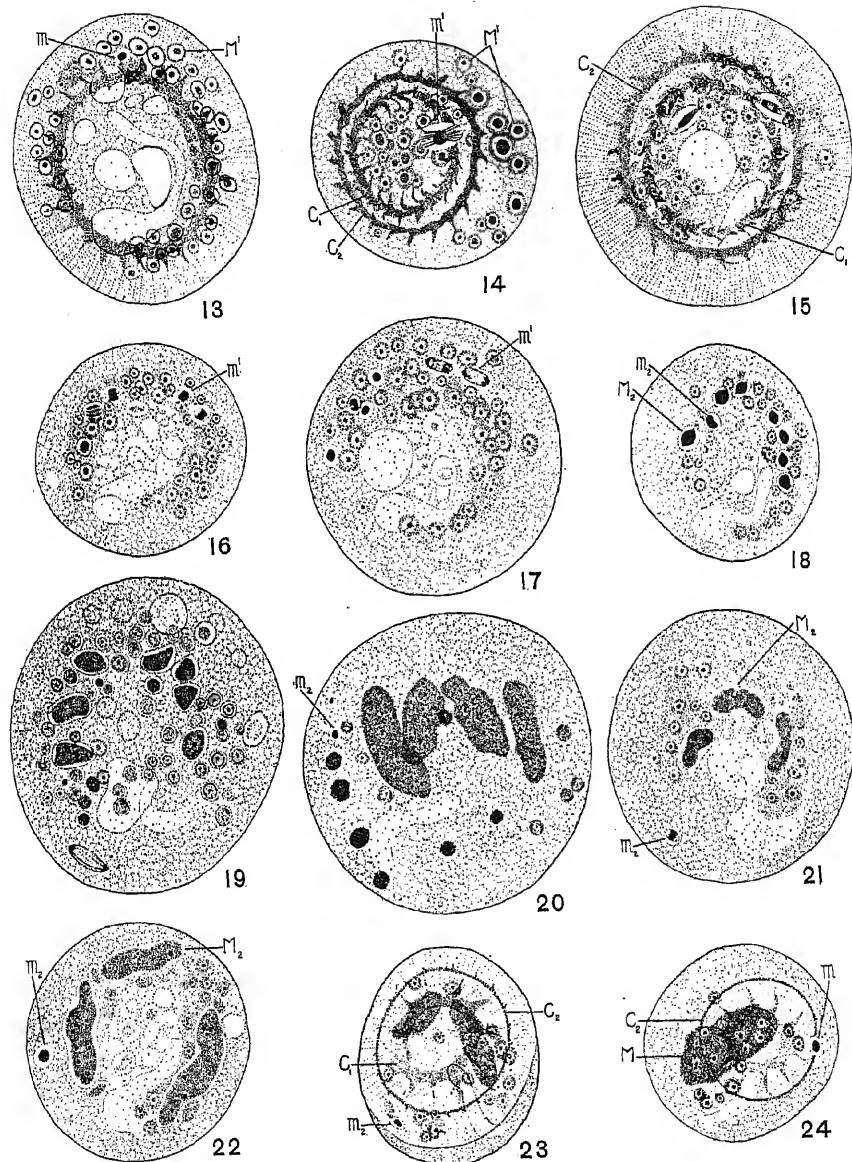
PLATE 3

Figures 25 to 36, variations in the process of endomixis.

25 Recent daughter resulting from a precocious endomictic division. Cytoplasmic strand showing. Twenty-two denticles in outer corona; ten in inner corona. Single micronucleus and about one-half the usual number of macronuclear fragments.

26 Eight nuclei, four large ones and four small ones which have just been formed.

27 Fourth division of the micronucleus; the two large nuclei in the upper right center have not yet divided. Eleven nuclei.

28 Sixteen-body stage; fourteen macronuclear anlagen and two functional micronuclei.

29 Sixteen-body stage; fifteen macronuclear anlagen and a prophase micronucleus.

30 Eight-body stage; seven macronuclear anlagen, a micronucleus, an inner partially resorbed corona of eight denticles, and a precoronal ring.

31 Twenty-seven nuclear bodies; three of these are significantly larger than the rest.

32 Twenty-three nuclear bodies; four of these are functional micronuclei. The three dark nuclei in the upper right center are probably degenerating. Disk side of the animal is turned away from the observer.

33 Nine-nucleate individual. Four nuclei are typical macronuclear anlagen, four are degenerating, and one is the functional micronucleus.

34 Ten nuclei; nine macronuclear anlagen and one functional micronucleus.

35 Eleven nuclei; four are functional macronuclear anlagen, the six in the upper right are degenerating, and the micronucleus is preparing for division.

36 Twelve-nucleate individual.
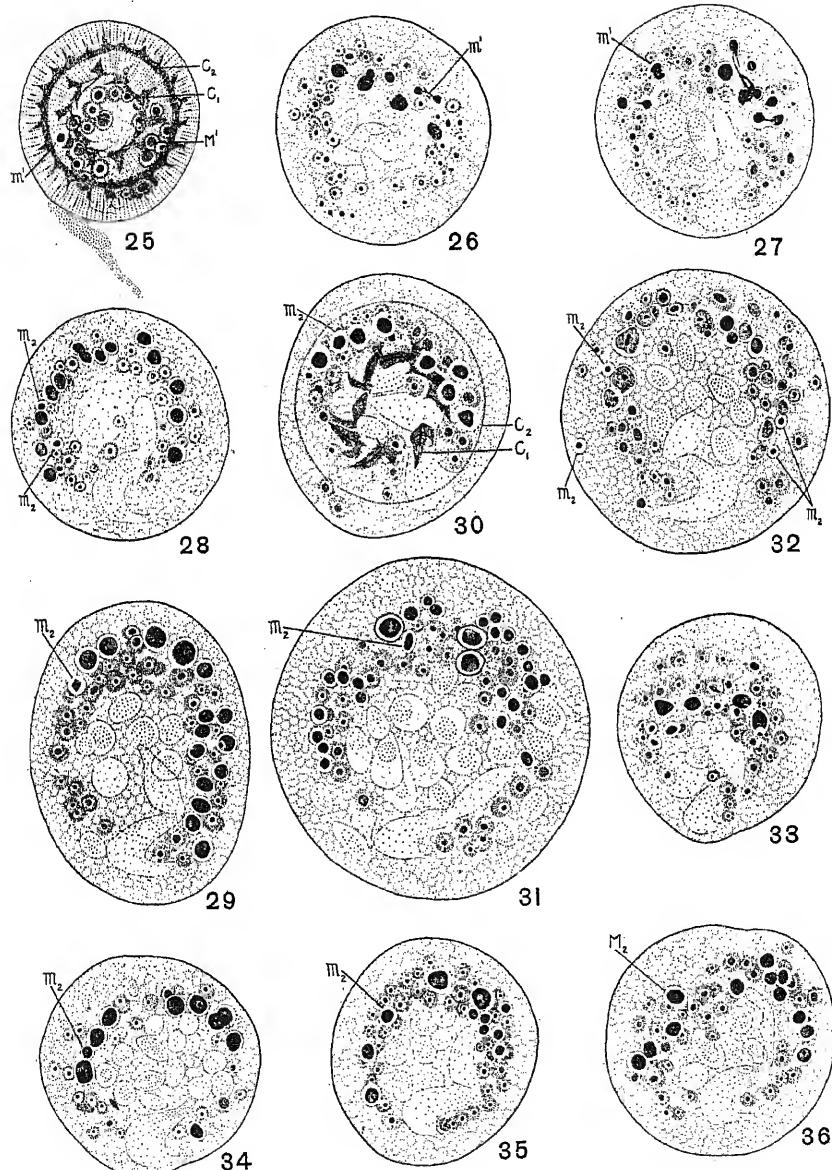
Figures 37 to 48, variations in the process of endomixis.

37 Twelve-body stage; four large macronuclear anlagen, six small nuclei, and two small micronuclei, just formed.

38 Thirteen-body stage. Three macronuclear anlagen are larger than the others. Micronucleus dividing. Centrodesmose visible.

39 Seven nuclei; one, in the upper part of the figure, beginning to differentiate into a macronuclear anlage; the other six are small and homogeneous.

40 Hypertrophied nucleus beginning to constrict.

41 Later stage in the division of a hypertrophied nucleus.

42 Two hypertrophied nuclei.

43 Second division of hypertrophied nucleus.

44 Four differentiating macronuclear anlagen and two coronas of approximately the same number of denticles.

45 Five macronuclear anlagen and a dividing micronucleus not in its usual position.

46 Three functional macronuclear anlagen, two degenerating macronuclear anlagen, and a micronucleus.

47 Two large macronuclear anlagen, a small macronuclear anlage, and a micronucleus.

48 Six macronuclear anlagen and two micronuclei.

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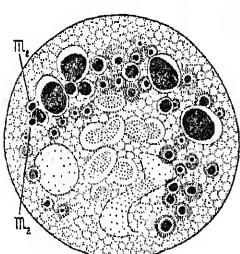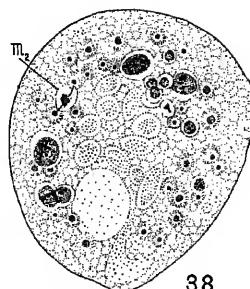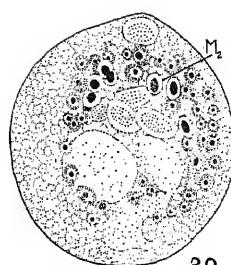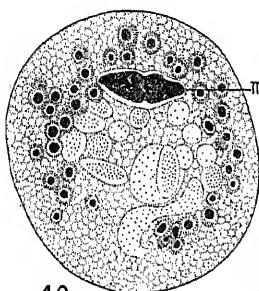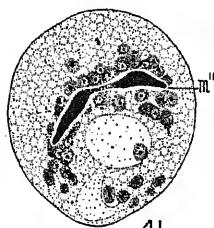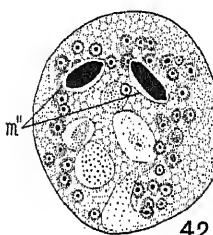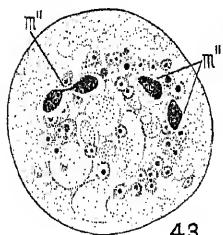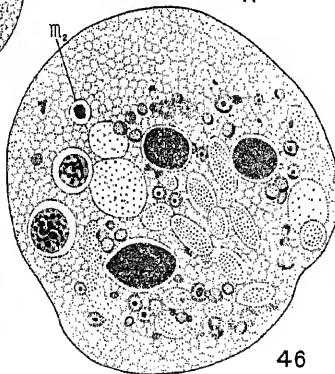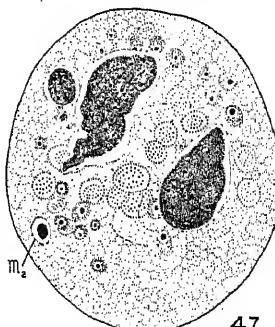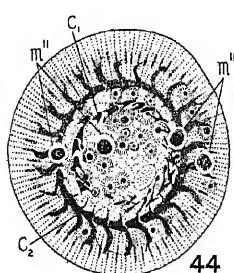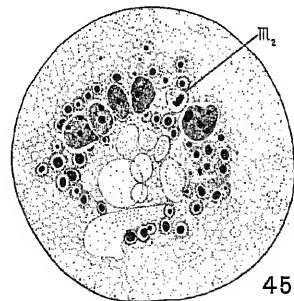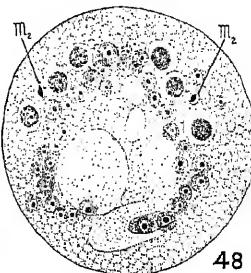
THE ANATOMY OF THE URINOGENITAL ORGANS OF THE MALE MYRMECOBIUS FASCIATUS

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FOUR PLATES (SIXTEEN FIGURES)

AUTHOR'S ABSTRACT

The urinogenital organs of Myrmecobius fasciatus conform to the marsupial type in both their anatomical characters and histological details. The external genitalia indicate a close relationship with the Dasyuridae.

The following account of the male urinogenital organs of Myrmecobius fasciatus has been worked out from a mature animal captured at Jarrawood, Western Australia, in December, 1925. This little animal has several descriptive names, which Wood Jones terms 'book' names, such as the marsupial anteater and the banded anteater, but its aboriginal name is the numbat.

As far as we are aware, the only reference to the anatomy of the male organs of the numbat consists of a short note in a paper by W. Leche ('91) devoted chiefly to myology. The anatomy of the female urinogenital system was worked out by J. P. Hill in 1900.

Some of the more obvious features are demonstrated by the two photographs of partial dissections in figures 1 and 2. Externally, the densely hairy scrotal sac is slightly bilobed and it hangs by a well-defined stalk in the characteristic marsupial position in front of the penis (fig. 1). Just anterior to the root of the tail is the well-developed colliculus urogenitalis; its summit bears a very shallow depression into which the cloaca opens. The perineum is a very thin transverse partition across the cloaca, separating the dorsal anal aperture from the ventral urinogenital sinus. The anus is oval and

widely open, as in Dasyurus. The wall of the urinogenital sinus continues forward toward the folded penis pocket which surrounds the base of the spiny penis, and the whole copulatory organ projects downward and forward from the body in the non-erectile condition of the penis. This is characteristic of the Dasyuridae; in other marsupials both the penis and the penis pocket are withdrawn into the body. In Myrmecobius there is no extra separate erectile body (corpus fibrosum) projecting from the penis pocket, such as is described in Dasyurus. The penis has a bifid tip (a feature characteristic of most polyprotodonts, but occurring also in diprotodonts); its free ends hang together, not widely separated as in Didelphys. The skin of the cleft glans penis is smooth, but the adjacent region, for a length of 0.7 cm., is beset by a well-developed series of stiff, backwardly projecting spines. On the ventral face a well-defined fold in the skin separates the glans from the spiny region and forms, as it were, a webbing at the base of the cleft. The proximal portion of the organ is covered with smooth loosely folded skin, devoid of hairs, forming the penis pocket.

Internally (fig. 2), the most striking feature of the system is the relatively enormous size of the anterior portion of the urethral tract or 'prostate.' This organ is a large, muscular, somewhat conical-shaped body, stretching from its base, on a level with the kidneys, to the pelvis. Though in all marsupials the urethral tract is characteristically long and wide, it appears to be especially well developed in Myrmecobius (fig. 3). Osgood ('21) described the prostate of Coenolestes as larger than in any other marsupial. He gives the ratio of the length of prostate to length of body in Coenolestes as 16:113. A similar comparison on this specimen of the numbat gives 30:105, so that in this case the organ is relatively much longer. There is no indication of a bilobed character such as occurs in the koala (Phascolarctos cinereus), where a longitudinal depression runs along the dorsal side.

The urethra may be divided into three regions, which vary in the structure of the wall. The conspicuous anterior region

of the urethra is often simply called the 'prostate.' But in monotremes and marsupials there is no prostate in the narrow sense; separate prostate glands with a common duct opening into the urinogenital canal do not occur. A mass of radially arranged tubular glands lies within the wall of the urethra and these open into its lumen by a number of separate apertures. This region is therefore termed the 'prostatic tract of the urethra.' Oudemans, however, considers that the so-called prostate is really a powerful development of urethral glands in both marsupials and monotremes. The lumen of the urethra is quite a small canal occupying the axis of the organ. The prostatic tract gradually narrows as it passes toward the pelvis, where a slight ridge marks the commencement of a thinner-walled membranous region, termed the pars membranacea, which is dorsally flexed and is protected by the symphysis pubis-ischio.

Paired prominent swellings (fig. 3 a), closely bound up in connective tissue and lying on either side of the urethra immediately posterior to the pelvis, form the colliculus urogenitalis and mark the commencement of the third division, the bulbous urethra, which is followed by the penis.

The kidneys have the usual form. The right kidney lies anterior to the left and is slightly larger. The adrenal bodies lie quite free from the kidneys on either side of the dorsal aorta, just anterior to the renal arteries; the left one is conspicuous in the photograph (fig. 2).

The urinary bladder opens into the prostatic tract dorsally; in the photograph (fig. 2) it is contracted and lies on the left side. In marsupials great differences occur in the part of the bladder which is transitional to the urinogenital canal. The following condition occurs in Myrmecobius (fig. 16). At the base of the bladder, the circular muscles increase in number and form a sphincter vesicae around a widely open lumen, which opens into a shallow chamber termed the collum vesicae. Its wall is thinner and less folded than that of the body of the bladder and it opens into the lumen of the urethra by a narrow canal. The paired ureters pass obliquely through the

wall of the collum vesicae and open quite close together on minute papillae on its floor. Immediately behind these apertures, the canal leading from the collum vesicae may be regarded as piercing the wall of the prostate and entering the urethra at right angles. This condition is markedly different from the arrangement in Didelphys and Thylacinus, where the bladder opens into a spindle-shaped expansion of the cranial end of the urethral canal. In Phascolomys there is a deep groove at the base of the bladder and no true neck is developed.

The wall of the bladder is made up of the usual three coats: mucous, muscular, and serous layers. The tunica mucosa consists of a dense connective-tissue layer (tunica propria) on which rests a layer of transitional epithelium. The tunica muscularis, which is loosely attached to it, is very richly supplied with blood vessels. An inner layer of longitudinal smooth fibers is surrounded by a layer of circularly arranged fibers.

Externally, the vasa deferentia appear to enter the urethra quite close to the ureters, but actually they run through its wall for a considerable distance and they open on a distinct median ridge (fig. 5) by a pair of minute pores situated about 4 mm. from the opening of the bladder. This ridge is the verumontanum or colliculus seminis. It is not equally developed in all marsupials. In Didelphys and Hypsiprymnus the vasa deferentia open on separate paired papillae directed caudad. In Dasyurus, Perameles, and the phalangers a colliculus seminis is present; it is especially well developed in Phascolomys, where it is recognizable as a conspicuous longitudinal fold, on the summit of which the vasa deferentia and some urethral glands open. In the koala a well-marked verumontanal eminence is described by Young, as well as an ill-defined depression on its summit termed the sinus pocularis. In Myrmecobius there does not appear to be any structure comparable to this sinus pocularis or vesicula prostatica. Both the verumontanal eminence and the sinus pocularis are entirely absent in Thylacinus, where the vasa deferentia open

by paired slit-like apertures (Cunningham). It appears that a sinus pocularis (uterus masculinus) is rarely developed in marsupials.

The testes (figs. 2 and 3 a), covered by their sheaths, lie within the scrotal sac with their median surfaces closely adpressed. They are ellipsoidal in shape (axes 1.8 by 1.2 cm.) and their efferent ducts pass into the epididymis from their median anterior margins.

Each testis and epididymis is inclosed within two transparent sheaths. The outer peritoneal sac, the tunica vaginalis (fig. 2, right testis), incloses the cremaster muscle, the sperm duct, and its associated blood vessels as they pass from the abdominal cavity into the scrotum. The testis and epididymis are attached to the inner surface of the scrotal sac only by thin strands of connective tissue running from the tunica vaginalis to its wall. There is no gubernaculum. The cremaster muscle is inserted into the dorsal face of the tunica vaginalis by a broad deltoid expansion of its fibers.

Beneath the tunica vaginalis lies the tunica albuginea (fig. 2, left testis). This coat forms the only attachment between the characteristically well-developed epididymis and the testis. The extent of the epididymis is illustrated in figure 2. The middle band-like corpus epididymis lies dorsal to the testis and connects the cap-like expansions, the caput and cauda epididymis, of which the cauda epididymis is the larger. The vas deferens, as it is differentiated from the caudal end, is much coiled till it reaches the abdominal orbit, within which it gradually straightens out except for a few twists and is only slightly thickened as it passes into the wall of the urethra. Vesiculae seminales are not developed.

The rest of the urinogenital organs consist of the accessory glands which open into the posterior portion of the urethra, the erectile tissues of the penis, and the muscles which control the movements of this organ. The total length of the urethra of this specimen is 9.25 cm. Its three regions already mentioned are distinguishable by the character of the wall. The anterior division is the glandular prostatic tract already de-

scribed. The middle membranous region has a thinner wall. It consists of that part of the urethra which is protected by the pelvic girdle, posterior to which it bends sharply ventrad and then is surrounded by the structures forming the third or bulbous region.

The structures connected with this postpelvic region of the urethra form the colliculus urogenitalis. In figure 2 they have been partially dissected out on the left of the specimen and are undisturbed on the right. They consist of three pairs of Cowper's glands, a pair of lymph glands, and two pairs of prominent muscular bulbs which inclose the 'heads' of the strands of erectile tissue which form the penis (fig. 3 a). The more anterior pair of these bulbs is formed by the ischio-cavernosi or erectores penis muscles which surround the bulbs of the paired corpora cavernosa. The posterior and more ventrally placed pair is formed by the bulbocavernosi muscles, which constitute thick powerful envelopes surrounding the double bulb of the corpus spongiosum (corpus cavernosum urethrae).

Structure of prostatic tract

Histological examination of the prostatic tract reveals a structure essentially similar to that of other marsupials, Halmaturus rufus, for example, described by Oudemans. In transverse section (fig. 5) a thin coat, a broad central glandular region, and the folded epithelial lining are readily distinguishable. The following layers are met with from the periphery to the lumen. The serous surface is covered by a delicate epithelium. Within this, there is a thin coat of smooth-muscle fibers; the peripheral fibers run circularly and surround the longitudinally arranged muscles. Within the muscle coat is a layer of dense connective tissue richly supplied with blood vessels. This connective tissue forms a cushion in which the expanded blind ends of the tubular glands are embedded, and it gives off slender projecting tags which lie between the glands and bind them together. The relative extent of the glandular portion is illustrated in

figure 5, and a small portion of the periphery of a section more highly magnified is illustrated in figure 7.

The glands are essentially of the compound tubular type, each consisting of several very elongated tubules joining up to form a common duct (fig. 8). Figure 9 illustrates a transverse section across the tubules about halfway along their length. In the region immediately surrounding the lumen of the urethra (fig. 10) the ducts pass through mesh-like connective tissue. The apertures of the ducts are very minute; they cannot be seen with the naked eye between the bases of the folds of the stratified epithelium which bounds the lumen.

The pars membranacea is not definitely marked off from the prostate, but is gradually differentiated from it by a relative increase in muscle and connective tissue, which latter projects inward and divides the glandular tissue into cords so that transverse sections show isolated groups of urethral glands opening into the lumen (fig. 11). The wall of the pars membranacea is further differentiated by a greater development of the connective tissue surrounding the muscle coat.

The bulbous urethra and its associated Cowper's glands

Most marsupials appear to possess three pairs of Cowper's glands, which vary in size and structure. Myrmecobius has three pairs (fig. 4 a), one of which is many times larger than the other two and even macroscopically a difference in the thickness of its muscle coat can be detected. In dissecting this part of the system three paired prominent muscular masses are exposed as soon as the connective tissues are removed from the colliculus urogenitalis. The largest of the three pairs of Cowper's glands is the most dorsally and laterally situated of these masses. (The other two masses are the bulbocavernosi and the ischiocavernosi muscles.) Their glandular structure is not obvious externally and their ducts (fig. 4 a) are entirely hidden by the ischiocavernosi and the bulbocavernosi muscles. A pair of superficially (i.e., ventrally) situated bean-shaped lobulated glands (fig. 3 a) lies in the notch between the bulbocavernosi and the largest Cow-

per's gland. The structure of this gland indicates that it is a lymph gland; neither dissection nor sections yielded a satisfactory duct. The second and third pairs of Cowper's glands are comparatively small. They lie dorsally in the notch on the first pair formed by the looping of the erectores penis (ischiocavernosi) muscles. Owing to comparatively thin walls, their glandular nature is obvious. These three pairs of glands have separate ducts which open quite close together into the dorsal wall of the urethra at the commencement of the bulbous urethra.

Structure of the Cowper's glands

The wall of the largest pair of Cowper's glands (fig. 14) consists of two irregularly arranged coats of striped-muscle fibers running roughly at right angles to each other; within this lies a layer of connective tissue which projects inward to form a framework for the support of the secreting epithelium. Both the muscular tissue and the connective tissue are richly supplied with blood vessels.

The secreting epithelium bounds a number of irregularly branched cavities which all lead into a large central space. The secreting cells are columnar, and their spherical nuclei lie near their bases. The short straight duct issues from the median border of the gland. The other two pairs are very similar to each other in general characteristics (fig. 12). Their walls are much thinner than those of gland *I*, but they consist of the same tissues. Though the cells of the secreting epithelium do not differ markedly from those of gland *I*, sections exhibit a greater density of glandular tissue; the acini of gland *I* are widely open spaces, but in the other two pairs of glands they are much smaller and more tubular. The spaces of all three pairs of glands contain a granular secretion (not represented in the figures) which is only lightly stained with Ehrlich's haematoxylin.

The urethra, after receiving the ducts of Cowper's glands, is surrounded by erectile tissue and muscles to form the bulbous urethra and penis. Both the corpus spongiosum and

the corpora cavernosa commence in double bulbs inclosed, respectively, by the paired bulbocavernosi and ischiocavernosi muscles already described (fig. 4 a). The bulbocavernosi lie slightly posterior to the ischiocavernosi and they are more prominent in a dissection from the ventral surface.

The corpus spongiosum (corpus cavernosum urethrae) is situated dorsally; it invests the urethra and forms the bulk of the glans penis. The paired corpora cavernosa (corpora cavernosa penis) run side by side for a short distance; then they almost fuse and thus surround the spongy body on its ventral and lateral faces. The whole structure is closely bound up by connective tissue and muscles.

Figure 13 illustrates a transverse section of the bulbous urethra in the position indicated by an arrow in figure 4 a. The lumen of the urethra is lined by columnar epithelium devoid of glands and it rests on a thin basement membrane. This is surrounded by mesh-like spongy tissue (corpus spongiosum) and then a coat of muscles running chiefly circularly. The cavernous tissue forms the bulk of the organ; strands of muscles project in from the sheath and form the framework within which the blood sinuses are contained. In Didelphys the corpora cavernosa remain separate, but in Myrmecobius, Phascogale, and some others they fuse to form one continuous structure.

The extremity of the penis is cleft for a length of about 5 mm. and at its base the urethral canal passes into a pair of grooves formed by the incurling of the inner edges of the cleft (fig. 15). The free tips are rather rounded than pointed and the smooth skin covering them is separated on the ventral face by a well-marked fold from the spiny region. In sections (fig. 6) the spines exhibit a core of very dense connective tissue bounded by a stratified epithelium exhibiting well-marked keratinization, the horny layer being somewhat thicker than the malpighian layer. The glans is formed of spongy tissue and the skin surrounding it consists of a thin layer of connective tissue and a stratified squamous epithelium only slightly keratinized.

The muscles of the penis

The muscles of the penis consist of four paired muscles and one accessory unpaired muscle. These are:

1. Sphincter cloacae, a broad band-like ring surrounding the cloaca and inclosing both the end of the rectum and the penis sheath. Though it is comparatively well developed in Myrmecobius fasciatus, it appears to have a much greater extent in some other marsupials (Owen), where it is described as inclosing also the bases of Cowper's glands. The cut ends of the sphincter cloacae are readily distinguishable in the photograph (fig. 2).

2. The obvious strand of muscle extending from the pelvis to the glans penis in the midventral line corresponds to the Y-shaped levatores penis muscle as typically developed in the opossum. But in Myrmecobius the arms of the Y are represented by a pair of extremely short fasciae attached to the ventral face of the ischiocavernosi near their stalks. From a firm attachment to the symphysis pubis a thick cord-like tendon extends to the fork of the Y. This is the ligamentum suspensorium penis (fig. 3 a) and it is not present in Didelphys. The relations of the pelvis to the colliculus urogenitalis and the penis are very similar in Myrmecobius and Dasyurus, and in the latter genus this ligament is a well-developed plate-like expansion. According to Broek ('10), it occurs in those forms in which the genitalia are situated far from the pelvis. It does not occur in Thylacinus.

3. The paired retractores penis muscles are band-like. They are attached to the sacrum, from which they turn outward and pass dorsal to the bulbo-urethra to the penis, along the dorsal surface of which they run side by side to their insertion into the base of the glans penis.

4. Each of the ischiocavernosi or erectores penis muscles is attached by a firm tendon to the center of the posterior border of the ischium. It then expands and makes a fleshy loop-like turn which incloses the bulb of the corpus cavernosum (crus penis) and is inserted into the stalk of this organ.

5. The bulbocavernosi have no connection with the pelvis. They constitute thick powerful muscular envelopes for the paired bulbs of the corpus spongiosum and they are attached to the fibrous capsules inclosing these bodies. Dorsally, the 'stalks' of these capsules are firmly united to each other and they are attached to the penis by loose connective tissue which also incloses the retractores penis muscles.

In many marsupials a varying number of rectal or anal glands is developed. A single conspicuous pair occurs in the female numbat, but they are absent in the male. Myrmecobius shares this sexual distribution with Coenolestes and some others. Rectal glands are also wanting in the males of Phascolarctos and Thylacinus. Though two pairs of rectal glands may occur, one pair is often absent. In all phalangers only the caudal pair is well developed; Halmaturus and Hypsiprymnus have only one pair in the male, but in Phalangista there are three pairs.

SUMMARY AND CONCLUSIONS

The internal urinogenital organs of Myrmecobius differ but slightly from the typical marsupial condition. They agree closely with Thylacinus, except that this genus lacks a verumontanal eminence and has only two pairs of Cowper's glands. On the whole, the structure of the male reproductive organs does not add anything to the interesting question of the relationships of the numbat. In regard to the external genitalia the investigation confirms several obvious features, already noted by Leche and Broek, which tend to ally Myrmecobius with the Dasyuridae. These are the permanently exserted condition of the penis and the high colliculus urogenitalis with its associated development of a ligamentum suspensorium penis. This ligament is cord-like in Myrmecobius and plate-like in Dasyurus.

The cloacal cavity is divided transversely by a partition, the perineum, as in Notoryctes, Perameles, etc., a condition regarded as primitive. Another strikingly primitive or generalized characteristic of Myrmecobius is the complete absence of a marsupium in any stage of the life-history.

AN ADDITIONAL NOTE CONCERNING RELATIONSHIPS

A great deal has been written about the relationships of Myrmecobius, chiefly based on a consideration of the dentition and foot structure. The foot structure of Myrmecobius is unique. In the Dasyuridae the hallux is much reduced in comparison with the Didelphidae. It is set at an angle, is clawless, and has a slightly swollen terminal pad. In the numbat the hallux is absent. Bensley, in his work on the relationships of the Australian marsupials, retained Myrmecobius in the family Dasyuridae, but he was unable to deduce any evidence of relationship from a study of the foot structure in either Myrmecobius or Thylacinus; these genera would not fit into a scheme of specialization of foot structure in the Dasyuridae, but rather indicated independent lines of development.

The dentition of the numbat has received more attention than any other part of its anatomy, on account of the extraordinary number and form of the molars. Bensley maintains that the peculiar dentition is not an indication of affinity with mesozoic mammals, but that it can be derived from a prototypal condition nearly realized by the smaller Dasyuridae. The presence of supernumerary molars is due to a repetition of teeth from the dental lamina and is connected with the extraordinary elongation of the jaw in accordance with the highly specialized ant-eating habits of the numbat.

Wood Jones adopts the foot structure as the primary criterion for the subdivision of the Didelphia. According to this system, both Myrmecobius and Thylacinus are raised to family status, along with Didelphys, Dasyurus, and Notoryctes, as the type genera of the five families which form the Didactyla.

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PLATE 1

EXPLANATION OF FIGURES

1 Myrmecobius fasciatus, ventral, external genitalia (the right tip of the penis has been removed).

2 Dissection of urinogenital system. Testis and colliculus urogenitalis dissected out on left side of specimen only.

PLATE 1

MALE GENITAL ORGANS OF MYRMECOBIUS
MAHALAH G. C. FORDHAM

3 a   Urinogenital system, ventral view.   × ⅝.

3 b   Kidney (horizontal section).

4 a   Urinogenital system removed from body; dorsal view.   On the right of the specimen the Cowper's glands have been removed, and the bulbocavernosus muscle slit up to expose bulb of the corpus spongiosum.   Ischiocavernosus muscle removed on left.   × 1½.

4 b   Diagram, median sagittal section through penis.

5   Transverse section through prostatic tract of urethra, cranial end.   × 6¼.

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6   Transverse section through spine skin of penis.   × 66.

7   Transverse section through prostatic tract of urethra, peripheral portion (see *A*, fig. 5).   × 135.

8   A gland from the prostatic tract.   × 27.

9   Section transverse to length of glands of prostatic tract (see *B*, fig. 5).   × 450.

10   Transverse section of prostatic tract of urethra, showing openings of glands into its lumen (see *C*, fig. 5).   × 133.

11   Transverse section of pars membranacea.   × 35.

12   Transverse section of Cowper's gland *II*.   × 105.

PLATE 1

MALE GENITAL ORGANS OF MYRMECOBIUS
MAHALAH G. C. FORDHAM
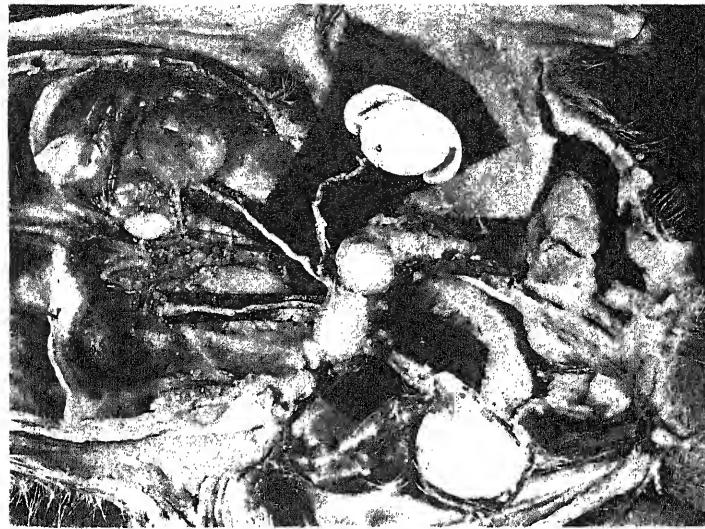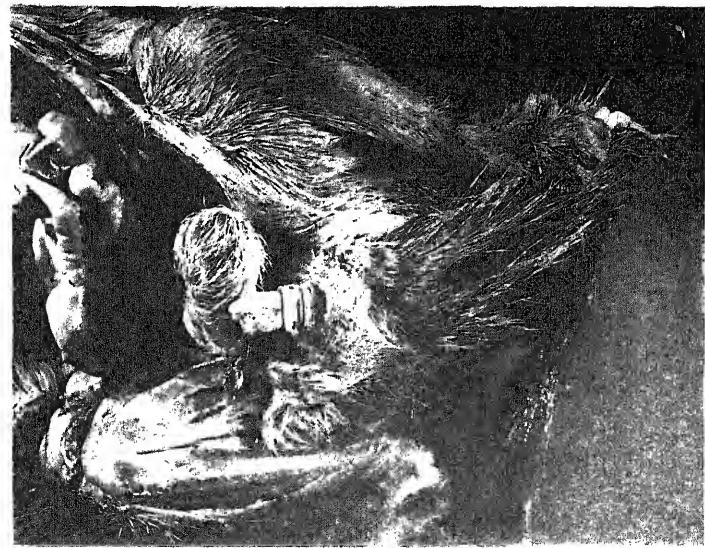
13 Transverse section of bulbous urethra (position indicated by arrow, fig. 4). × 23.

14 Transverse section of Cowper's gland *I*. × 96.

15 Transverse section of one half of bifid glans penis. × 15.

16 Diagrammatic sagittal section of bladder and prostate.

3a.

3b.

4a.

4b.

5.

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